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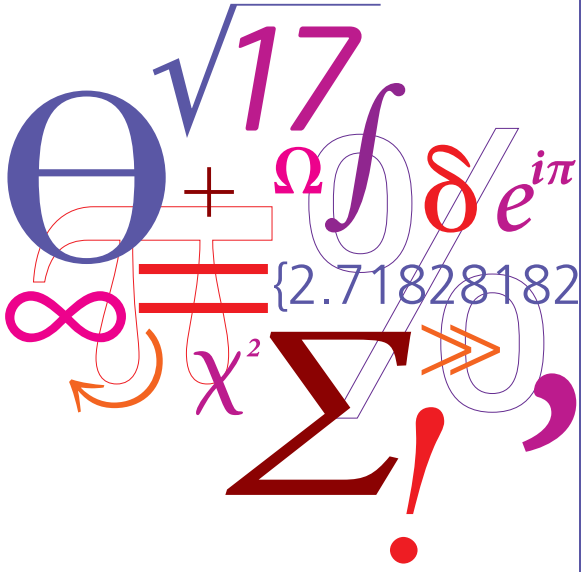
Technical University of Denmark

Controlled release of environmentally friendly antifouling agents from marine coatings



Stefan Møller Olsen
2009

DTU Chemical Engineering
Department of Chemical and Biochemical Engineering

Controlled release of environmentally friendly antifouling agents from marine coatings

Stefan Møller Olsen

Ph.D. Thesis

Marts 2009

Department of Chemical and Biochemical Engineering

CHEC Research group

Technical University of Denmark

DK-2800 Kgs. Lyngby

Denmark

Hyldest til hverdagen

Jeg holder af hverdagen
Mest af alt holder jeg af hverdagen
Den langsomme opvågnen til den kendte udsigt
der alligevel ikke er helt så kendt
Familiens på en gang fortrolige of efter søvnens fjernhed
fremmede ansigter

Morgenkyssene
Postens smæld i entréen
Kaffelugten
Den rituelle vandring til købmanden om hjørnet efter mælk,
cigaretter, aviser –
Jeg holder af hverdagen
Selv gennem alle dens irritationer
Bussen, der skramler udenfor på gaden
Telefonen, der uafladelig forstyrrer det smukkeste, blankeste
stillestående ingenting i mit akvarium
Fuglene, der pipper fra deres bur
Den gamle nabo, der ser forbi
Ungen, der skal hentes i børnehaven netop som man er kommet i
gang
Den konstante indkøbsliste i jakkelommen
med sine faste krav om kød, kartofler, kaffe og kiks
Den lille hurtige på den lokale
når vi alle sammen mødes med indkøbsposer og tørrer sved af
panderne
-
Jeg holder af hverdagen
Dagsordenen
Også den biologiske
De uundgåelige procedurer i badet og på toilettet
Den obligatoriske barbermaskine
De breve, der skal skrives
Huslejeopkrævningen
Afstemningen af checkhæftet
Opvasken
Erkendelsen af at være udgået for bleer eller tape –
Jeg holder af hverdagen
Ikke i modsætning til fest og farver, tjald og balfaldera
Det skal til
Med alle sine efterladte slagger
Så meget usagt og tilnærmelsesvist
vævende og hængende i luften bagefter
Som en art psykiske tømmermænd
Kun hverdagens morgenkaffe kan kurere
Fint nok med fester! Al plads for euforien!
Lad de tusinde perler boble
Men hvilken lykke så bagefter at lægge sig
i hvilens og hverdagens seng
til den kendte og alligevel ikke så kendte samme udsigt

Jeg holder af hverdagen
Jeg er helt vild med den
Hold da helt ferie hvor jeg holder af hverdagen
Jeg holder stinkende meget af hverdagen

Celebration of everyday life

I like everyday life
Most of all I like everyday life
The slow awakening to the familiar view
that is not so familiar after all
The familiar, but from the distance of sleep so strange faces of
the family

The morning kisses
The sound of mail dropping
The smell of coffee
The ritual walk to the grocery around the corner for milk,
cigarettes, papers –
I like everyday life
Even through all its annoyances
The buss that rattles outside on the street
The phone that unstoppably interrupts the most beautiful,
shining stagnant nothing in my aquarium
The birds that chirps from their cage
The old neighbour that drops by
The kid that has to be picked up in kindergarten just as you were
getting started
The constant shopping list in your pocket
with its continuous demands for meet, milk, macaroni and
mushrooms
The quick one at the local joint
when we all meet with our shopping backs to mop our brow

I like everyday life
The agenda
Also the biological
The inevitable procedure in the shower and on the lavatory
The obligatory razor
The letters that need writing
Rent payment
Balancing of the cheque book
Dish washing
The recognition of having run out of diapers or tape
I like everyday life
Not in opposition to music, dance, pot and party balloons
It is needed
With all the mud left behind
So much unsaid and approximate
waffling and left in the air to dry
A kind of mental hangover
That all can be cured by everyday's morning coffee
Parties are fine! Make room for euphoria!
Let those thousands of pearls bubble
But what a joy to lie down afterwards
in the bed of rest and everyday life
to the same familiar and yet not so familiar view

I like everyday life
I am crazy about it
Holy smoke, I like everyday life
I like everyday life bloody much

Dan Turéll

Preface

This dissertation is the outcome of three years research carried out partly at CHEC research group under the department of Chemical and Biochemical Engineering at the Technical University of Denmark, and partly at Hempel A/S. Associated Professor Søren Kiil, Ph.D Lars Thorslund Pedersen, M.Sc. Merete H Hermann (formerly Laursen) and Professor Kim Dam-Johansen have supervised the work during these three years research towards the Ph.D. degree, and the work was funded by the Danish Technical Research Council, Hempel A/S and the Danish Agency for Science Technology and Innovation

If the research described in this thesis is logically presented, and the information is easy to access and understand, the reader should thank associate professor Søren Kiil. He has been untiring in his work on improving the paper manuscripts. His thoroughness and astonishing carefulness for details has been an inspiration that has not only improved this work, but also taught me valuable lessons for my work to come. Lars Thorslund Pedersen has been an important driver for the progress of the work during the past three years. His realistic and always sensible reasoning has been of great importance to keep progressing. In addition, it has been great fun to discuss everything from oversize beavers to science and odd combinations of sports and those conversations have been of great importance for me to keep up the spirit when the results did not come. Together, Søren and Lars have first and foremost been a team of very challenging supervisors and good company, and it has been three extremely fruitful years for me. Also thanks to Merete H Hermann, for always taking the discussions and also for taking part actively in the project. I am very thankful to Kim Dam-Johansen for having gotten the opportunity and for him keeping track in a busy schedule.

The research has been done in collaboration with Danisco, and the fruitful meetings and good discussions with Jakob Broberg Kristensen, and Brian Søgaard Laursen are greatly appreciated. It is impressing that it has been so easy communicating across scientific boarders, and I believe it to be your responsibility primarily.

I need to thank Ajish John whom I supervised during his Master's thesis. Besides being a very nice acquaintance he was hard working and provided key contribution to this thesis. Also Inesa Tautkeviciute is thanked for her thorough and independent work during her individual course.

I probably should not start mentioning names, but people have been so extremely kind to me when I have been knocking on their doors with stupid questions and weird requirements. At

Hempel, an office with Vibeke Stendal Larsen (thanks for all the massages) Claus Weinell, Andreas Paulsen, Shaloumeh Mehlat Oxfeldt, Lotte Unn and Lars Thorslund Pedersen has always been entertaining to be a part of, and I have enjoyed every day. The rest of the group, Hans Jørgen Hansen, Karl-Johan Hansen, Mette Brandi, and Erik Graversen have always treated me with more kindness than I think I deserve. I am also thankful towards the whole antifouling/fouling release/fouling control group. Daniel Schønemann has always been willing to discuss and answer questions, and I have very much enjoyed the short time we got to cooperate. Ciaran Dunbar always made room for me and my projects, though I was not always able to fill out all the requests. Lena respectfully told me when I had not cleaned sufficiently thorough, and Fabian Barrientos, and Dorthe Hillerup have always taken time to enlighten me when I did not understand. Thank you to Ulrik Bork, Anders Blom and Peter Thorlaksen for being flexible and making room for me in Jyllinge, and for not shaking your head at my crazy ideas. I would also like to thank Karen Eng in the analysis laboratory, Engku Osman in Singapore, and Antoni Sanchez in Spain for being patient with me. Also thanks to Thomas Beck for allowing me to extent my stay at Hempel, and last, but not least thank you to Diego Meseguer Yebra for being helpful, and good company, and for setting the bar so high that there was no way I could reach it. I improved very much in my effort.

At DTU I need to thank the entire CHEC group for treating me as one of your own even though I spent more than half of my time elsewhere. I very much enjoyed sharing office with Jimmy Andersen and Pavle Andric in my most frequent time at DTU, and thank you to Per Aggerholm Sørensen for the many discussions and your willingness to help. Thank you to Hanne Frøkiær for letting me borrow the micro plate reader time after time.

To my friends, I would like to say thank you for your patience with me, especially the past months have been lonely, but the knowledge of how easy it is to return to being Stefan after the hard work would end has been a key contributor for me finishing this.

Finally to my family and my amazing girlfriend, if you do not know my appreciation of you I have failed utterly and nothing I could write here should make sense. I would therefore like to dedicate this to the everyday; the everydays that have passed during these three years, those that passed before, and those that will pass in the future. My everydays I spent with you.

Summary

Controlled release of environmentally friendly antifouling agents from marine coatings

This work on controlled release of environmentally benign antifouling agents from marine coatings is primarily concerned with hydrogen peroxide as the environmentally friendly, active antifouling agent. Hydrogen peroxide is considered environmentally friendly, as it decomposes rapidly into water and oxygen after having been released to the seawater. However, several organisms are equipped with catalase, an enzyme used to protect against hydrogen peroxide, and therefore the effect of hydrogen peroxide as antifouling agent is an important subject for investigation.

The overall aim of this work has been to evaluate the effect of a two-enzyme system producing hydrogen peroxide from starch. It is therefore considered of prime priority to be able to conclude on the feasibility of antifouling coatings based on this technology.

In chapter one, an introduction to fouling, the marine environment and the working mechanisms of antifouling coatings will be given. Chapter two provides an overview of antifouling based on enzymatic catalysis as has been reported in patents and peer reviewed literature. Chapter three describes the application of inorganic peroxides as precursors for hydrogen peroxide in an antifouling coating. Chapter four is about one of the aspects in developing an antifouling coating based on enzymatic production of hydrogen peroxide from starch, namely the achievement of a self-polishing antifouling coating independent on seawater soluble metal oxides or salts. Chapter five evaluates the antifouling effect of enzyme mediated hydrogen peroxide ultimately originating from starch in the coating, and chapter six concerns the work done to determine the effect of hydrogen peroxide when released from a surface, in a stable manner.

Inorganic peroxides have been tried as new antifouling coating ingredients, and the release of hydrogen peroxide from inorganic peroxide containing coatings has been tested for antifouling efficiency. When released from a zinc oxide/peroxide containing coating, only 7.5 µg hydrogen peroxide /(cm^2·day) improves the antifouling effect of the underlying, zinc oxide-based, polishing coating. This coating however, is not slime free, and nor is it comparable to the antifouling performance of a cuprous oxide containing commercially available antifouling coating.

As a consequence of working on starch and enzyme-based hydrogen peroxide release, a coating composition capable of polishing without containing seawater-soluble metal oxides or salts has been developed. Whereas coatings containing starch only, do not polish, polishing rates of

between 7 and 10 µm/month are achieved when starch and the starch degrading enzyme glucoamylase is added to the coatings in substantial amounts. The coating that is based on slow dissolution of starch by the enzymatic degradation into glucose is believed to be able to deliver antifouling agents in a stable manner, due to its polishing mechanism.

Enzyme mediated degradation of starch into glucose followed by the (also enzyme mediated) oxidation of glucose to gluconolactone under the release of hydrogen peroxide, has been tested under ocean seawater conditions. The antifouling effect of hydrogen peroxide released from enzymatic conversion of starch via glucose (by the enzyme glucoamylase) to hydrogen peroxide (by the enzyme hexose oxidase) is dependent on the climatic conditions on the site of immersion. The enzyme/starch based coatings, loose antifouling activity rapidly under warmer climatic conditions. An environment of high temperature and intense biofouling pressure causes the coatings to foul greatly within eight to fourteen weeks. However, under less intense conditions, the antifouling performance of the same coating is exceeding that of a cuprous oxide based reference. The temperature dependent difference in antifouling activity is due to a temperature dependent loss of hydrogen peroxide release rates, which is ultimately caused by a hydrogen peroxide dependent decay of glucoamylase. The potential of enzyme-mediated hydrogen peroxide is therefore depending on whether glucoamylase can be stabilised to retain its activity in the presence of hydrogen peroxide.

A controlled release assay has been developed based on cellulose acetate membranes, and the assay has been used to establish the required release rate of hydrogen peroxide needed to keep a surface free of fouling. When released alone from a non-polishing surface of cellulose acetate coated kraft paper, a release rates between 225 and 2800 µg/(cm^2·day) of hydrogen peroxide is required to keep the surfaces completely free of fouling during nine weeks immersion in Jyllinge Harbour, Denmark. This value is beyond the reach of any of the means described here to provide hydrogen peroxide in-situ in an antifouling coating.

To summarize, if an enzyme-based hydrogen peroxide releasing coating is successful in converting all the starch into hydrogen peroxide during one year of operation, it is doubtful that hydrogen peroxide in the obtainable quantities alone can carry an antifouling effect. To be a successful antifoulant, it must therefore be a part of a larger, more diversified, fouling protection system.

Resumé (Summary in Danish)

Kontrolleret udludning af miljøvenlige biocider fra antifoulingmalinger

Denne afhandling omhandler kontrolleret udludning af miljøvenlige biocider fra antifouling malinger. Det meste af det arbejde, der er beskrevet heri, er det miljøvenlige biocid, der beskrives hydrogenperoxid. Hydrogenperoxid kan betragtes som værende miljøvenligt idet det hurtigt henfalder til vand og ilt efter at være udludet i havvand. Desværre forekommer hydrogenperoxid naturtligt i havmiljøet og mange organismer er sågar udstyret med enzymer, der beskytter dem mod hydrogenperoxid. Måling af effekten af hydrogenperoxid som antifouling agent er derfor en central del i dette arbejde.

Det overordnede mål er at kunne evaluere antifouling effekten af en maling, der ved hjælp af to enzymer omdanner stivelse til hydrogenperoxid.

I første kapitel vil der blive givet en introduktion til fouling, det marine miljø, og mekanismerne involveret i biocid frigivelse fra antifouling malinger. Andet kapitel giver et overblik over de, i artikler og patenter, beskrevne tiltag til enzymbaseret antifouling. I kapitel tre er anvendeligheden af uorganiske peroxider som prekursor for hydrogenperoxid i antifouling malinger beskrevet, og kapitel fire beskriver udviklingen af en antifouling maling, der polerer som følge af kontrolleret enzymatisk nedbrydning af vanduopløseligt stivelse til vandopløseligt glukose. Kapitel fem evaluerer antifouling effekten af enzym medieret udludning af hydrogen peroxid fra stivelsesholdig antifouling maling, og sjette kapitel omhandler de eksperimenter, der er blevet udført med henblik på at teste forskellige frigivelseshastighed af hydrogenperoxid på biofouling.

Uorganiske peroxider er blevet testet som nye antifouling maling ingredienser, og frigivelsen af hydrogenperoxid fra malinger indeholdende disse nye ingredienser er blevet testet som antifouling maling. Frigivet fra en zinkoxid/peroxid holdig maling, 7.5 µg hydrogen peroxid/(cm^{2}·dag) forbedrer antifoulingeffekten signifikant i forhold til en zincoxid baseret reference maling. Disse malinger er dog ikke slim fri og kan heller ikke sammenlignes med kobberholdige antifoulingmalinger.

Som en konsekvens af at have arbejdet på stivelse og enzym-baseret hydrogenperoxid udludning, er der blevet udviklet en maling, der kan polere uden at indeholde store mængder metaloxid. Malingen afhænger af den enzymkontrollerede nedbrydelse af stivelse til vandopløseligt glukose, og poleringshastigheder mellem syv og ti µm/måned er opnået. Dette betyder, at malingen er et muligt medie for kontrolleret frigivelse af andre biocider.

Enzymbaseret nedbrydning af stivelse til glukose, efterfulgt af en (også enzymbaseret) oxidering af glukose til glukonolaktone under frigivelse af hydrogen peroxid, er blevet testet for antifoulingeffekt under forskellige klimatiske forhold. Effekten afhænger i høj grad af temperaturen på stedet. Således begror malingerne meget hurtigt under varme forhold. Under tempererede forhold har malingerne (andetsteds) vist sig at have en begroningshindrende effekt, der overgår antifoulingeffekten af kommercielle referencer. Forskellen i effekten kan spores tilbage til temperaturen, og mere konkret er det glukosefrigivelsen der tabes hurtigere under varme forhold. Dette skyldes at hydrogenperoxidet der bliver dannet i den anden enzymreaktion fremskynder nedbrydningen af det første enzym (glucoamylase). Potentialet af enzymbaseret hydrogenperoxid udludning som antifouling afhænger derfor af om glucoamylase kan stabiliseres således, at det kan bibeholde sin aktivitet tilstrækkelig længe under tilstedeværelse af hydrogenperoxid

Et kontrolleret frigivelses assay, baseret på anvendelse af celluloseacetat membraner til at kontrollere diffusionen af et biocid, er blevet udviklet. Assayet er det første af sin slags, der kan evaluere effekten af biocider som funktion af frigivelseshastigheden. Effekten af forskellige frigivelseshastigheder af hydrogenperoxid på biofouling er blevet testet, og hydrogenperoxid frigivet som eneste biocid fra en ikke-polerende overflade kræver en frigivelseshastighed på et sted mellem 225 og 2800 µg/(cm^2·dag) for at holde overfladen fri for biofouling, når denne er placeret i Jyllinge havn i Danmark i løbet af ni uger om efteråret. Disse værdier er udenfor rækkevidde for de to metoder, der her har været benyttet til at frigive hydrogenperoxid fra en antifouling maling, enzymatisk fra stivelse og hydrolyse af uorganiske peroxider.

Det vil sige, at hvis en enzym baseret, hydrogenperoxid udludende maling formår at producere hydrogenperoxid af al det, i malingen, tilgængelige stivelse, vil det være tvivlsomt hvor vidt, den effekt, der kan opnås er tilstrækkelig til at bære en tilfredsstillende antifouling effekt alene. For at blive en tilstrækkelig potent miljøvenlig antifouling maling, skal enzymbaseret hydrogenperoxid udludning kun indgå som en del af et mere bredspektret og diverst antifouling system.

Table of contents

1. Chapter one – Fouling, the marine environment and antifouling coatings

This survey is divided into four sections. First will be given an introduction to biofouling in the marine environment, and then the technology of antifouling coatings will be described. Biocidal compounds, commonly, and potentially utilised in antifouling coatings are described in section three, and section four provides a discussion on the scope of environmentally friendly antifouling. Supplementary information regarding hydrogen peroxide as antifoulant is available in Appendix I.

The content of this chapter is available as an internal report titled marine fouling and antifouling, a literature survey. (Athors: Olsen S M, Kiil S, Thorslund L T)

Biofouling

In this section, the reasons for - and mechanisms of - biofouling will be described, the composition of biofilms, and the marine environment will also be touched upon, and a brief description of the most common fouling organisms will be provided.

Mechanisms of biofouling

Fouling is inevitable for almost any material inserted into seawater (Rittschof 2001). The common development of fouling can be divided into three steps: Molecular fouling, microfouling and macrofouling (Abarzua and Jakubowski 1995). A schematic illustration of one mechanism and constituents of a fouling layer is given in Figure 1.1.

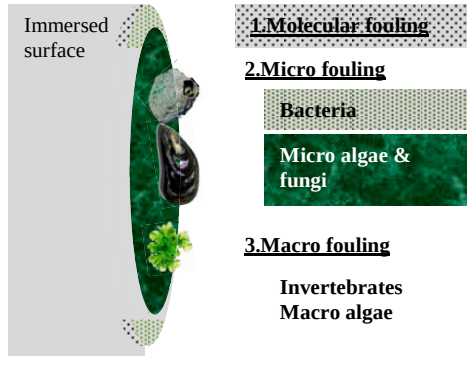


Figure 1.1: Fouling layers of common fouling mechanisms.

Following introduction into seawater organic polymers accumulates on the surface of the material, creating a conditioning film (Compere et al. 2001). This is called molecular fouling, the primary cause of which is adsorption of proteins and polysaccharides originating from the marine biosphere. Microfouling consists of two steps, primary and secondary colonization (Abarzua and Jakubowski 1995). The primary colonizers is made up of bacteria and diatoms in that order, they adsorb to the conditioning layer via Brownian motion, electrostatic interaction, gravity and van der Waal forces, this adsorption is reversible, as the bacteria can easily be removed. Many primary colonizers can create extracellular polysaccharides and by adhesion attach themselves to the conditioning film irreversibly (Abarzua and Jakubowski 1995). Secondary colonizers consists of spores of macroalgae and protozae, these are still considered a part of the microfilm. An interesting feature of microfouling is that the mass of the microfilm progresses nonlinearly (Souza and Bhosle 2003). Macrofouling is partly consisting of the secondary colonizers (the spores of macrofoulers), and tertiary colonizers. The tertiary colonizers are the larvae of the macrofouling organisms, which makes the distinction between micro- and macro-foulers difficult (Abarzua and Jakubowski 1995). Bacteria seem to appear within hours of immersion in seawater, diatoms within days, secondary colonizers in a week, and tertiary colonizers in 2-3 weeks (Abarzua and Jakubowski 1995). This generalized picture of the development of biofouling is called succesional fouling (Rittschof 2001). It has been generally assumed that this order of events is obligate (Cooksey and Wigglesworth-Cooksey 1995), thus if one could prevent microfouling, macrofouling would also be inhibited. This is not the case. Attachment of macrofoulers to microfilm-free surfaces is generally applied in bioassays testing new biocides, barnacles are widely used. Clare et al. (1992) describes a probability driven fouling mechanism as a supplement to the succesional fouling mechanism depicted in Figure 1.1. Probability driven fouling is dependent on the physiological state of the organism and the properties of the surface, as well as the number of propagules (individual-producing units) available (Rittschof 2001). Some macrofouling species use one of the two techniques alone; others use combinations thereof dependent on the conditions (Rittschof 2001). A schematic illustration of probability driven fouling is given in Figure 1.2. Not only the balance between free and fouled individuals should be taken into account, also the interactions between free individuals of different species are influencing the process (Clare et al. 1992).

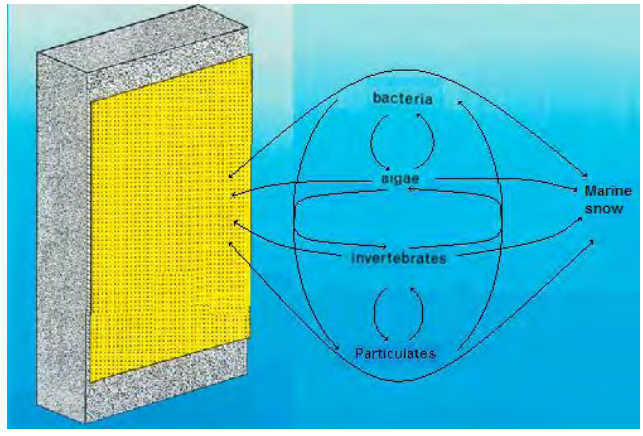


Figure 1.2: Dynamic model of fouling. Modified from (Clare et al. 1992).

Quorum sensing

As described above bacteria and other micro organisms benefit greatly of attaching to surfaces. Therefore it is of great interest, how the organisms sense the surfaces. The phenomenon of Quorum sensing has been known for long, but its widespread use in microbiology is only recently understood (Miller and Bassler 2001). Quorum sensing is a molecular language of binary extent. It is believed that bacteria uses this way of communicating to act as a multi-cellular community (Geske et al. 2005), as such quorum sensing is involved when bacteria is creating biofilms. The molecule that is responsible for this communication differs between species, but in general gram-positive bacteria use processed oligo-peptides, and gram-negative bacteria use acylated homoserinelactones (AHL) (the gram terminology is a physiological distinction between bacteria). According to Corpe (1973) (Dempsey 1981), the majority (85-90%) of the fouling bacteria are gram-negative.

The quorum sensing regulation of the LuxI and LuxR proteins are very common in Gram-negative bacteria. The mechanism follows a general development: The LuxI protein synthesise the AHL that is distributed evenly over the cell membrane. When the bulkconcentration of AHL reaches a critical border value, it binds to the LuxR protein. This complex binds to a promoter and activates transcription of the gene coding for LuxI. This results in a positive feedback mechanism, where the AHL concentration is increasing rapidly. Other specific genes are also up regulated as a consequence of the increased AHL concentration (Miller and Bassler 2001). When the autoinducer is produced, it diffuses away from the cell and is diluted by a factor of the radius cubed. If the

density of bacteria is high, or if the bacteria are close to a surface, the concentration of the autoinducer molecule (AHL) is relatively high. If it exceeds the critical concentration, the positive feedback mechanism is induced. In this manner the bacteria becomes capable of sensing a surface or a dense population. Another gene regulated as a consequence of quorum sensing is responsible for creating exo-polymeric substances, allowing the organism to adhere to the surface sensed (Decho 2000). The mechanism is depicted in Figure 1.3.

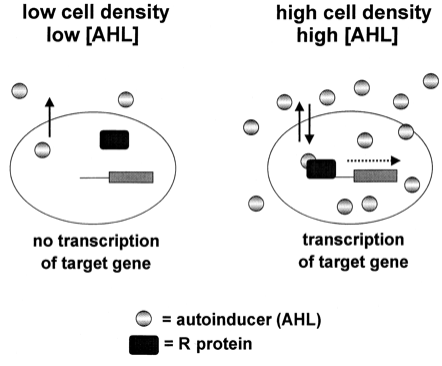


Figure 1.3: Mechanism on quorum sensing. From (de Kievit and Iglewski 2000) with permission.

The composition of biofilms

Biofilms differ to a great extent from the individual bacteria. In fact, biofilm bacterium may be considered as a distinct phenotype of the bacterium genome (Souza and Bhosle 2003). Firstly a great amount of the film is made up by extracellular polymeric substances (EPS). These polymers consist of polysaccharides and proteins primarily, and their function is to glue the bacteria to the surroundings. Between 75 and 90 mass percent of the dry biofilm is made up of EPS (Costerton 1999). Secondly the bacterial community in a biofilm can be made up of a great variety of species, with different EPS. The surroundings of one bacterium can therefore differ from the surroundings of one of the same species, in a different micro colony (Costerton 1999).

According to Costerton (1999), the microfilm is made up of units of micro colonies. These micro colonies are often mushroom-shaped and with pores that allows water and solutes to penetrate rapidly. The structure of the biofilm depends amongst others on the substrate concentration (Wimpenny et al. 2000). Beside the mushroom structure, the traditional view of the microfilm as a planar homogeneous structure is described, along with a third structure called the heterogeneous

mosaic model (Walker and Keevil 1992). This structure is made up of stacks of colonies upon a layer of cells held together by EPS (Wimpenny et al. 2000). Allison (2003) describes three similar microfilm models, but he also concludes that the composure of the film is variable over time. As the nutrient level alters, so does the biofilm (Allison 2003).

It can be argued that the three forms of biofilm structure primarily differ in surface area, the two first being the opposite extremes. Therefore if the micro colony benefits from a large surface area (i.e. nutrient rich, toxic-free and favourable oxygen conditions) the structure will be mushroom shaped. If the opposite is the case, the planar homogenous structure will dominate. This relationship is very complex as a result of the many parameters involved, and their interrelations.

The arrangement of micro organisms in microcolonies, gives rise to gradients. Nutrients, gases, and metabolites can have varying concentrations over a very small distance and the buffer capacity of the matrix can alter the pH from one micro colony to the next. (Allison 2003). Therefore aerobic and anaerobic conditions can occur, as well as nutrient rich and nutrient poor microenvironment can be found within a short range of each other. This gives rise to local differences in diversity, type of bacteria, and structure of the matrix (Allison 2003).

In addition to the complexity of the microfilm, the macrofouling community is also very inhomogeneous. It is made up of larvae of macrofoulers, mussels, barnacles, seaweed etc. Some of these are predators, they too will be selective. Possible toxic metabolites from the microfilm bacteria, aerobic/anaerobic conditions and pH are other parameters affecting the distribution of the macrofouling species and thereby adding to the heterogeneity on a macroscale (Allison 2003). The complexity of a fouling film is increased with the increasing mass of the film, and therefore with the exposure time to seawater. As a further contribution to complexity, the fouling film can enhance possibility of corrosion (Melo and Bott 1997), leading to alterations in morphology and increased concentrations of heavy metal ions.

Further complexity arises when antifouling coatings are considered. Dempsey (1981) describes a difference in the morphology and bacterial composition of surfaces painted with antifouling coatings. Comparing antifouling coatings containing triphenyltinfluoride, cuprous oxide and no toxin, it is found that the microfilm of the two former consists primarily of bacteria, whereas two coatings containing no biocide has a very diverse fouling community, consisting of bacteria, diatoms, choanoflagellates and protozoa (Dempsey 1981). Jackson & Jones (1988) report a difference between the microorganisms fouling on a coating containing organotin and cuprous oxide respectively. Fouling by diatoms and filamentous algae on coating based on cuprous oxide to

be increased, and the use of organotin results in fouling by green algae and amphipod (Jackson and Jones 1988).

Variables affecting biofilms

The morphology of a biofilm is dependent on the temperature, pH, nutrient availability and water flow rate (Melo and Bott 1997). As the relationship seems very complex due to the complexity of a biofilm, the exact nature of these dependencies is not well determined. The following descriptions is therefore only brief and of superficial extent.

Nutritional variation

Three parameters can be used to predict bacteria-based biofilm formation as a consequence of the nutrients level in the water.

1. Metabolically active bacteria have a great tendency to adhere to surfaces.
2. Biofilm growth is limited by the amount of nutrients available for cell replication and EPS production.
3. In nutrient deficient water, bacteria do not adhere to surfaces, biofilm formation is limited to places where nutrients are present. (Souza and Bhosle 2003)

High nutrient levels have been shown to produce an open structure of the biofilm, and lower nutritional values appear to cause flat, more closed biofilm structures (Melo and Bott 1997). These observations are, however, not always reproduced (Souza and Bhosle 2003), which is probably because not only nutritional value determines whether biofilm bacteria benefit from a large surface area. Toxins, oxygen concentration, and optimum temperature, may also have an effect. The inversely proportional relationship between nutrients and oxygen availability in a living matrix, can further complex the correlations.

Turbulence

In general, biofouling is reduced for higher velocity of the water or the body moving in water (Melo and Bott 1997), but the opposite tendency has also been documented (Melo et al. 1988). A rule of thumb for turbular heat exchangers is that the fouling activity is reduced for velocities greater than 1 m/s (Melo and Bott 1997). Furthermore, the morphology of the biofilm is dependent on the velocity

of the moving water. Density of biofilms of pseudomonas fluorescens has been shown to increase with water velocities (Melo and Bott 1997).

Surface properties

There has been a large amount of investigations of the effect of surface –smoothness and –energy on biofouling. The commercial success of the fouling release coatings on fast ships (cf section 2) paints a clear picture of the effectiveness of combining a smooth surface of low energy with high ship velocity.

In general a smooth surface has less fouling than a rough one (Melo and Bott 1997). This is due to the lower surface area of a smooth surface (Melo et al. 1988), which causes the forces between the fouling species and the substratum to be weaker, but the smoothness of the underlying substratum has no impact after the initial layer of fouling film has settled. On a microscopic scale, the topography has been shown to have an effect on biofouling, both qualitatively and quantitatively. Bers and Wahl have examined the microtopography of the surface of several marine species (Bers and Wahl 2004). They report that the surface of Cancer pagurus (an edible crab) is inhibiting macrofoulers. And the microtopography of eggcase (a protective capsule of some animals) of Ophiura texturata (related to sea stars) is inhibiting microfoulers. The surface structure of the former is described as spicules with diameter of 2 µm. And the latter is described as knoblike structures with diameters of 30-50 µm. The fouling inhibiting effect is however decreasing over time (Bers and Wahl 2004). This consideration gives rise to speculations concerning the microfouling species alterations of the topography, and whether or not their presence is obligate. Bers and Wahl presented two other possible explanations for the decreasing inhibitory effect of the microtexture. The selectivity of larvae can decrease with decreasing space and time passed. Furthermore, footprints from macrofoulers examining the surface can accumulate and, if plenty enough, their effect can overwrite the repulsive effect of the microtopography (Bers and Wahl 2004).

Temperature

The optimum temperature for biofilm growth, in general, is around 40^{o}C, until this temperature the growth rate increases linearly, and for higher temperatures, it decreases linearly (Egan 1987). Optimum temperature of biofouling is a complex number, depending on the optimal temperature of

the species constituting the fouling community. In general the optimum temperature for microbial species in water is between 20 and 45^{o}C (Melo et al. 1988).

The temperature range in seawater is between -2 and 30^{o}C (Anderson 2003). This means that the fouling intensity differs between degrees of altitude, but almost not on a longituditional gradient, due to the almost evenly distributed surface temperature (when not considering the ocean currents such as the Gulf stream).

pH

As it is the case for temperature, the pH optimum of a fouling community is determined by its constituents. For seawater, the pH is very stable at pH 8 ± 0.4. It has been stated, that controlling the pH either above or below a critical acidity/alkalinity would inhibit fouling effectively. But manipulation of pH of a coating in contact with seawater is next to impossible because of the buffering of seawater and the effect of dilution.

The marine environment

The water of the oceans and major seas can be divided into an upper mixed layer, a main thermocline and the deep water. The distinction of the three layers is by temperature; therefore the location of their borders depends on the degree of latitude. At tropic regions, the upper mixed layer penetrates to around 200 meters, this border rises as sun intensity decreases, and in Polar Regions the temperature is uniform throughout the water column. The Main thermocline reaches 1000 meters at tropical area, but also diminishes in the Polar Regions (Anderson 2003).
It is in the upper layer that the major temperature variations occur. The temperature in surface waters can vary between -2 and above 30 centigrade. The main thermocline is determined by a rapid decrease in temperature. In this volume, the temperature decreases from that of the surface water to the deep water. The deep water temperature is constant between -2 and +5 centigrade. Whereas the surface water makes up around 2 % of the total seawater volume, the deep water constitutes 80 % (Anderson 2003).

The salinity of ocean waters is also dependent on the depth. For surface water it can vary between 3.3 and 3.8 % and for deep water it is even more stable at 3.47 +/- 1 % (Yebra et al. 2004). The salt-composition is also a very constant figure for ocean waters (Anderson 2003). Because the major alterations of the salt content are dilution by rainfall and concentration due to evaporation

(Yebra et al. 2004), the major variations occur in the upper layer. The species making up the seawater salinity is given in Table 1.1 together with some common trace elements.

Table 1.1: Average concentration of the most abundant elements in seawater. Adapted from (Grasshoff 1976).

Element	Main chemical speciation	Concentration (µg/L)
Chloride	Cl^-	$18.8 \cdot 10^6$
Sodium	Na^+	$10.77 \cdot 10^6$
Magnesium	Mg^{2+}	$12.9 \cdot 10^5$
Sulphur	SO_4^{2-}, $NaSO_4^-$	$9.05 \cdot 10^5$
Calcium	Ca^{2+}	$4.12 \cdot 10^5$
Potassium	K^+	$3.8 \cdot 10^5$
Nitrogen	NO_3^-, NO_2^-, NH_4^+	$1,5 \cdot 10^5$
Strontium	Sr^{2+}	$8 \cdot 10^4$
Bromide	Br^-	$6.7 \cdot 10^4$
Carbon	HCO_3^-, CO_3^{2-}, CO_2	$2.8 \cdot 10^4$
Silicon	$Si(OH)_4$	$2 \cdot 10^4$
Boron	$B(OH)_3$,$B(OH)_4^-$	$4.44 \cdot 10^3$
Fluoride	F^-, MgF^+	$1.3 \cdot 10^3$
Phosphor	HPO_4^{2-}, PO_4^{3-}, $H_2PO_4^-$	60
Iodide	IO_3^-, I^-	60
Molybdenum	MoO_4^{2-}	10
Zinc	$ZnOH^+$, Zn^{2+}, $ZnCO_3$	4.9
Arsenic	$HAsO_4^{2-}$, $H_2AsO_4^-$	3.7
Uranium	$UO_2(CO_3)_2^{4-}$	3.2
Vanadium	$H_2VO_4^-$, HVO_4^{2-}	2.5
Aluminium	$Al(OH)_4^-$	2
Iron	$Fe(OH)_2^+$	2
Nickel	Ni^{2+}	1.7
Titanium	$Ti(OH)_4$	1
Copper	$CuCO_3$	0.5
Chromium	$Cr(OH)_3$, CrO_4^{2+}	0.3
Manganese	Mn^{2+}, $MnCl^+$	0.2
Cadmium	$CdCl_2$	0.1
Tungsten	WO_4^{2-}	0.1
Cobalt	Co^{2+}	0.05
Mercury	$HgCl_4^{2-}$, $HgCl_2$	0.03
Lead	$PbCO_3$, $PB(CO_3)_2^{2-}$	0.03
Silver	$AgCl_3^{2-}$	0.03

Seawater pH is also very stable. In the upper mixed layer, the water is in equilibrium with atmospheric carbon dioxide creating a buffer. The pH is slightly alkaline lying in the range of 8.0 – 8.3. Deviations from these values occur locally due to overwhelming microbial activity removing carbon dioxide or creating acidic or alkaline metabolites, at shores near embankments of rivers, or as a consequence of human activity (Yebra et al. 2004).

The main gasses of interest are oxygen and carbon dioxide. Oxygen is taken up by the marine biosphere, and the carbon dioxide is used by the photosynthetic plants. Therefore the content and composition of gasses dissolved in the water varies, dependent primarily on the life type and its amount in the water (Anderson 2003). Colder water can hold more dissolved gasses than warmer,

but if the gasses are used biologically, the concentration can be zero. Therefore the content of oxygen in seawater can vary between 0 and 20 ppm, where 20 ppm is only reached where the number of sea plants is high, and it is sunny and windy (Anderson 2003).

Plant nutrients, nitrogen, phosphorous and potassium tend to sink to the bottom water as dead material or faeces, decomposition of this happens at the deeper seawater. The major plant growth however, happens in the surface water where the sun is. This separates the plants from the nutrients by the termocline. At some locations an upward stream (upwelling) is returning the nutrients to the surface waters, giving rise to an intensive plant growth (Anderson 2003).

Consequences of marine biofouling

The economic consequences of biofouling on ships hulls were estimated in 1989 by Milne (Townsin 2003). He estimated the savings on a world scale due to effective antifouling coatings. The savings were divided into four groups; the result of his estimations is presented in Table 1.2.

Table 1.2: sources and estimated savings due to application of antifouling coating. Adapted from (Townsin 2003).

Cause of saving	Estimated value (million US Dollars)
Reduced ship frictional resistance	720
Higher dry dock intervals	409
Lower dry dock costs	800
Indirect savings	1080

The figures presented in Table 1.2 add up to 3 billion US Dollars annually (Townsin 2003). In the study fuel savings of $7.36 \cdot 10^6$ tonnes is estimated. This correlates to a reduced emission of green house gases by $20 \cdot 10^6$ tonnes (Townsin 2003). Figures from 2000 states a 40 % increase in fuel consumption and up to 77 % increase in overall costs (Yebra et al. 2004).

On a smaller scale, the frictional resistance of fouling has been determined in more than one occasion, Schultz (2004) measured the frictional resistance of a clean and fouled self polishing copper-based antifouling coating. He found that the frictional resistance of a biofouled coating was around 100 times larger than that of a clean coating (Schultz 2004), and based on laboratory and full scale experiments, microbial biofilms are shown to be capable of increasing skin friction drag significantly (Schultz and Swain 2000). Measurements of drag resistance of antifouling coating samples, freshly applied, aged coatings and larger irregularities are also described in the literature (Weinell et al. 2003). The authors conclude that the irregularities on ships hull (weld seams etc.) contributes more to the frictional resistance, than irregularities in the coating system. Larger scale

irregularities such as macro fouling are reported to be the most important contributor to drag resistance, leaving micro and macro irregularities negligible (Weinell et al. 2003). However, Schultz (2007) show that whereas calcareous fouling will result in a change in total drag resistance of up to 80%, heavy slime will contribute as much as 20%.

Important fouling species

From the perspective of an antifouling coating manufacturer, the most important macrofouling species involves the following list: Barnacles, mussels, polychaete worms, bryozoans and seaweed.

Barnacles

Barnacles are probably the most important fouling species in this content. Pictures of common barnacles are presented in Figure 1.4. More than 1000 species of barnacles are known today (Enxyclopædia Britannica 2008a). Adult barnacles are usually fastened to a surface; it has eight calcareous plates, making up a volcano-like shell as is seen in Figure 1.4.

Barnacles are hermaphrodites, meaning that individuals contain both male and female sex organs; reproduction is done between adjacent adults. A single adult barnacle can release as much as 10,000 larvae. The larvae (nauplii) are released to the water as plankton. The nauplius stage as plankton is making up the first of two stages in barnacle settlement. The nauplii are transported by current or other transport mechanisms around in the sea. The nauplius stage takes about two weeks, and then it metamorphoses into a non-feeding, swimming cyprid larva. Because they are not feeding, cyprids must settle on surfaces within a resonably time frame. When this is done, they metamorphose into juvenile barnacle; they then develop the calcareous armor plates characteristic for the adult barnacle. The adult barnacles are fixed to the spot, to which they settle for the rest of their life. Therefore barnacles are dependent on the water mobility due to current or tidal waters to be provided with nutrients and planktonic food (Encyclopædia Britannica 2008a). Some species can survive only two hours in seawater a day.

Figure 1.4: Left: Barnacles on a blue mussel. Right; Barnacles on the bottom of a ships keel (from Johannesen 2008 with permission).

The most common species used in settlement assays during development of antifouling substances, is the barnacle *Balanus amphitrite*. The adult *Balanus amphitrite* has a diameter of approximately 1.5cm. It is found in intertidal fouling communities of harbors and embayment, attached to any hard surface (Hawaii Biological survey 2001).

Mussels

Mussels are another common fouling species that live in all types of waters. They are found on both ship hulls and used commonly in laboratory antifouling assays. Figure 1.5 shows a picture of the common blue mussel.

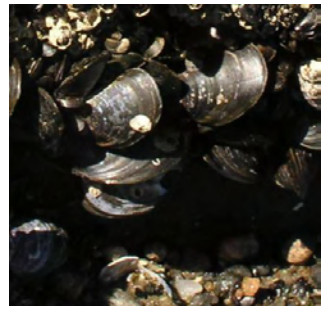


Figure 1.5: Blue mussels (from Johannesen 2008 with permission).

Mussels are generally gonochoristic (reproduction occurs between two distinct sexes). Fertilisation occurs outside the body, and the eggs develop into a larval stage that may drift for up to six months before settling on a hard surface. If the environment is suitable, the development into an adult

mussel will proceed. Mussels are adhering themselves to surfaces by use of byssus (long protinacious threadlike fibers) secreted from the byssus gland in the foot. The byssus hardens when in contact with water, which makes the adherent very tough (Encyclopædia Britannica 2008b). Mussels feed on plankton, by filtering seawater, and several mammals are feeding on mussels. The mussel load is heaviest in the low and mid intertidal zone in temperate seas, and the great variety of mussels makes them abundant in many regions (freshwater, salty waters, quiet waters etc) (Encyclopædia Britannica 2008b).The blue mussel, Mytilus edulis is the most used mussel in antifouling assays.

Polychaetes

Polychaetes are a class of (annelid) worms that generally live in the marine environment. They are sometimes called bristle worms because they have outgrowths that bear many bristles; these are made up of chitin. There are around 10,000 living species in the family, varying to a great extent regarding of physiology. Free living and stationary species are found, amongst others. The latter is of interest from an antifouling perspective. The large variations between different species are illustrated in Figure 1.6. The large feather-like structures, on the left hand side of Figure 1.6 are used both as gills and paddles (Myers 2001).

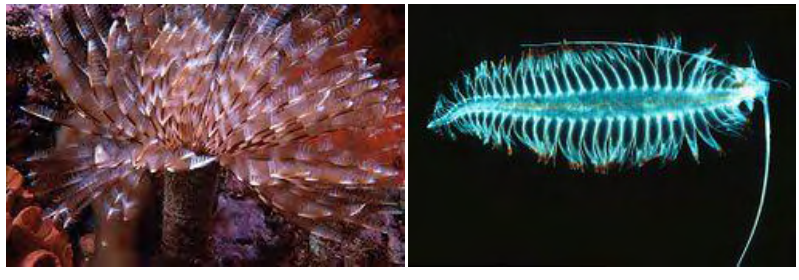


Figure 1.6: Left: Eudictylia polymorpha. Right: Planktonic polychaete (published under the GNU Free documentation license).

Bryozoans

Bryozoans are very small animals living in a colony made up of calcium carbonate or chitin. They live on any type of hard surfaces, primarily in the marine environment and preferably, but not exclusively in tropical waters. They are for the most parts immobile in their colonies, but exceptions occur. The colonies can be of metric sizes, though the individuals are generally within the range of one millimetre in diameter. The individuals in a colony have specific functions, such as food gathering, colony strengthening or cleaning. The development of a bryozoan colony can result in a number of different shapes and figures. The skeleton of the colony have small openings containing the zooid (the individual living member of the colony). The zooid feed on diatoms and unicellular algae, but because of their small size they have no circulatory or nervous system. In the innermost parts of the colony, the access to food and nutrients is limited. The zooids in these parts are dormant. Bryozoans are hermaphrodites, and reproduction can happen asexually and sexually (Encyclopædia Britannica 2008c). Figure 1.7 shows a saltwater and a freshwater bryozoan.

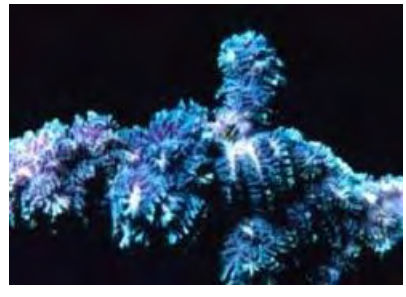


Figure 1.7: The bryozoans *Costazia costazi*.

Seaweeds

Seaweeds are macroscopic multicellular algae. They are divided into red, green and brown algae. The structure of seaweeds resembles terrestrial plants, with a body, a leaf and stem like structure amongst others. Attachment of seaweed is facilitated by the holdfast. The sea lettuce, Ulva linza is common seaweed, reported as fouling organism, and used in antifouling assays. Figure 1.8 shows a picture of common seaweeds attached to a rock (Encyclopædia Britannica 2008d).

Figure 1.8: Seaweed fastened to rocks (published under the GNU Free documentation license).

Other fouling organisms

Other fouling organisms that have been studied involve Diatoms (Navicula perminuta (Pettitt et al. 2004)), bryozoan, alga, and hydroid larva. Bacteria living in the marine environment involve proteobacteria, Acinetobacter and Cobetia marina, amongst others. The amount and diversity of fouling was evaluated by Woods Hole Oceanographic Institute in 1952. The list is presented in Table 1.3.

Table 1.3 Species diversity of the fouling community.

Plants	Number of reported fouling species
Bacteria	37
Fungi	14
Algae	563
Animals	**Number of reported fouling species**
Protozoa	99
Porifera (sponge)	33
Coelenterata (Hydrozoa etc.)	286
Platyhelminthes (flatworms)	12
Nemertea	11
Rotifera	5
Bryozoa	139
Brachiopoda	1
Annelida	108
Arthropoda (Barnacles etc.)	292
Mollusca	212
Echinodermata	19
Chordata	127

From Table 1.3, it can be seen that Arthropoda, Mollusca and Coelenterata are the most repreented classes. The two former include mussels and barnacles, which is consistent with the common expectations to fouling. The latter includes Ctenophore and Cnidaria and sea anemones amongst others. It is also seen that a large number of algae species are present as foulers.

Table 1.4: The glue of common fouling species. From Bonaventura et al. (1991).

Group	Class	Pre-settlement adhesive	Adhesive
Bacteria	Pseudomonas	Protein and polysaccharides ?	Polysaccharides and proteins
Coelenterata	Hydrozoa	Nematocysts	Mucopolysaccharides (probably)
Algae	Entermopha		Glycol-proteins
Bryozoa	Cheilostomat	Acidic mucopolysaccharides	Protein and sulfonated acidic mucopolysaccharide
Mollusca		Nucuous threads	Quinone cross-linked protein (from phenoles (Hellio et al. 2000))
Arthropoda		Secretion from cyprid antennule	Cyprid: alkaline protein (may be cross-linked with phenol) Adult: Acidic protein (may be cross-linked with phenols)
Annelida	Spirobinae	Mucuous threads	Sulfonated polysaccharides
Chordata	Tunicata		Protein with many S-S and S-H groups and asulfonated acidic mucopolysaccharide

The exopolymeric substances used by some of the relevant marine species are listed in the patent of Bonaventura and co-workers from.The substances are presented in Table 1.4.

In Table 1.4, it is seen that barnacles (members of arthropodes), use primarily protein as adhesive in their fouling mechanism. The blue mussel, is a mollusk, and is according to Table 1.4 settling by the adherent effects of cross linked proteins. The crosslink is according to Hellio et al. provided by reaction of amine residues with quinone. The quinone is produced by oxidation of phenols to chatecols and ultimately quinone, phenoloxidase is catalyzing the oxidation taking place in the byssus gland of the mussel (Hellio et al. 2000).

Antifouling coatings

In this section, the historical approaches to antifouling are described. This is followed by a description of the common constituents in antifouling coatings. The mechanisms of biocide release are then illustrated. Also alternative approaches to antifouling are described, and finally mathematical modelling of antifouling coatings is reviewed.

Historical perspective

Humans have been combating fouling for more than 2000 years. Pitch, wax, tar and asphalt are included in some of the earliest reporting of ship hull-coatings, applied by the ancient Greeks (Yebra et al. 2004). Also lead sheathing applied by copper nails was used by Romans and Greeks.

Scraping of the filth from the ships sides can be considered a very direct antifouling approach. Such initiative was described in the 1st century, to prepare the ships to sail more easily. Pitch was used in the 13th through 15th century. Up to the 18th century, lead was probably the most commonly used sheathing. Leonardo da Vinci has been mentioned in an antifouling-context, as he invented a rolling mill capable of making lead sheathing. The use of copper started in 1758. It became a great success though the antifouling mechanism was not understood for the first fifty years. Copper induces corrosion of iron, therefore the introduction of iron ships resulted in a renewed focus on antifouling coatings. Different alternatives (zinc, lead, nickel, arsenic, galvanised iron) were tried, along with coatings intended to seal the iron from the copper coating (Yebra et al. 2004).

Paints were introduced in the 19th century. They were primarily based on the biocidal effect of cuprous-, arsenic-, and mercury oxides. And binders constituted primarily of linseed oil, tar or plant resins. Various copper based coatings applied on a shielding coat were applied despite short operational time, inefficiency, and the fact that they were costly (Yebra et al. 2004).
Mercury oxide and zinc oxide composed the biocides of coatings with lifetimes of 9 months that were successful in the beginning of the last century. It was initially utilizing gum-shellac as a binder, but as it became increasingly costly, the binder was substituted with rosin (from trees). Cold plastic coatings, as opposed to the hot plastic coatings, which are based on mixtures of rosin and copper compounds, increased the operational time of the coating by a factor of two (Yebra et al. 2004). Organotin was introduced following the Second World War, together with synthetic resins. The results were improved mechanical strength and antifouling efficiency (Yebra et al. 2004). From the 1960's, tributyltin (TBT) were thought to have solved the fouling problem for good. But increasing environmental concern led to legislation that put an end to the regime of TBT during the beginning of the 3rd millennium (cf. section four).

Coating constituents

Paint is generally constituted of binders, solvents, pigments and additives. This is also the case for antifouling coatings. However, the pigments and binders usually differ from their conventional counterparts. As it will be described later, the pigments of major interest are the toxicological ones. The binders differ based on the antifouling mechanism (and thus by choice of antifouling coating type). In the following a brief description of the most common antifouling coating binders will be followed by a listing of pigments and additives. Note that the discussion concerned with biocides (toxicological pigments) is reserved for section three.

Binders

Antifouling coatings are generally physically drying coatings, which mean that only evaporation of solvent/diluent is resulting in formation of the film. Table 1.5 lists the most conventional binders in modern antifouling coating systems. The list is not necessarily exhaustive, but is thought to provide an overview of the types of binders utilized in commercial antifouling coatings.

Table 1.5: List of binders used in antifouling coatings.

Coating type	Characteristics	Binder
Insoluble matrix	High molecular weight	Insoluble vinyl Acrylic rubber Chlorinated rubber Eopoxy
Soluble matrix	Water soluble	Gum Rosin Wood rosin Tall oil rosin
Other		Bituminous binder Cellulose derivatives
Chemical polishing	Water-reacting	Silylated acrylic TBT-acrylic

The use of different binders is due to a variety of challenges. Firstly the properties of the binders are determining the characteristics of the coating. The working mechanism of the coating composed of the different binders is presented in Table 1.5. The mechanisms listed in the table are described more thoroughly in a later paragraph. As the border between the different mechanisms is vague, distinction is not always possible. Patenting has caused the coating manufactures to think creatively to develop suitable binder material. This is resulting in a great amount of different binders varying more or less in structure.

Rosin is a mixture of solid organic molecules. It has a glass transition temperature around 30 ºC and is brittle and friable. In average the molecular weight is 300 g/mol. It is soluble in aliphatic as well as aromatic solvents, together with chlorinated hydrocarbons, esters and ethers (Class 2000). Rosin is divided into natural and modified rosin.

Natural rosin is obtained from pine trees, primarily longleaf Pinus palustris, slash Pinus ellioti and loblolly pine Pinus taeda. Depending on their source, and the way of isolation, the natural rosin is divided into three groups; gum rosin, wood rosin and tall oil rosin.

Gum rosin is obtained by cutting V-shaped wounds in the bark of living trees. The rosin is collected in buckets, and painting the wounds with sulphuric acid promotes production. The collected oleoresin is composed of turpentine and gum rosin, which is separated by distillation.

Wood rosin is isolated from pieces of aged pine stumps. The stumps are cut into wood chips that are extracted with an organic solvent. Aliphatic or aromatic petroleum or ketones are used. Extracts are separated on behalf of volatility and solvents are recovered. The separated extracts are usually refined further by use of selective solvents (Class 2000).

Tall oil rosin is a by-product of paper manufacturing. Wood chips are treated with a combination of sodium hydroxide and sodium sulphide, under high pressure and elevated temperature. Sodium salts of rosin acids and fatty acids are removed, treated with sulphuric acid and separated by fractional distillation (Class 2000).

The distinction between the three different kind of natural rosin is not only based on production, the content and distribution of acid resin is also varying between the three types. This means that the physical and chemical characteristics are differing (Class 2000).

The rosin acids making up the resins can be divided into abietic-type acids and pimaric-type acids. The difference being a methyl and vinyl group, attached in the former case and an isopropyl group in the latter case. The most common resin acids are presented in Figure 1.9. In general natural rosin comprises 90% resin acids and 10% neutral compounds that are esters or decarboxylated derivatives of resin acids. In the case of tall oil, the commercial products also contain 2-5% fatty acids (Class 2000).

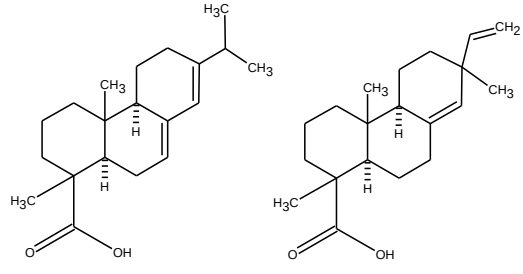


Figure 1.9: The two most common natural rosins. Left: abietic acid, Right: Isopimaric acid.

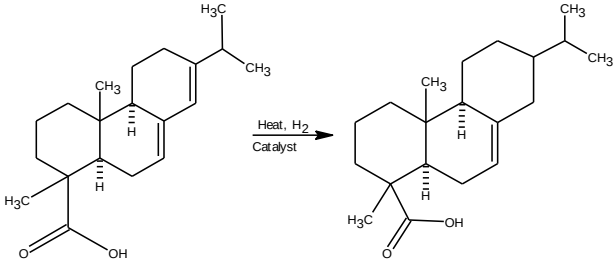
As will be described more detailed later, the success of rosin in antifouling coatings is due to its (limited) solubility in water. Parameters affecting the solubility are (Yebra 2005):

- pH of the water. Due to its acidic nature, rosin is more easily solubilised under alkaline conditions.
- NaCl concentration in the water. Increasing the sodium content increases the solubility.

- Calcium and magnisium ions. Resinates of calcium and magnesium are insoluble in water. Existence of these species reduces solubility of rosin.
- Oxidation is increasing the number of hydrophilic groups in the molecule, and thus solubility.

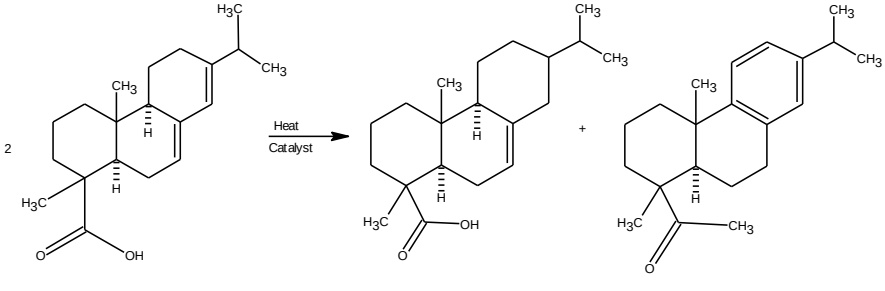
Especially the (conjugated) double bonds of the resin acids compromises their stability (Class 2000), therefore effort has been put into removing the double bonds.

Reduction with hydrogen (hydrogenation) over a suitable (metallic) catalyst is a common way of removing double bonds in organic chemistry, and is also applied in rosin chemistry (Class 2000). The reduction needs elevated temperatures to be carried out with a satisfactory yield.



(1.1)

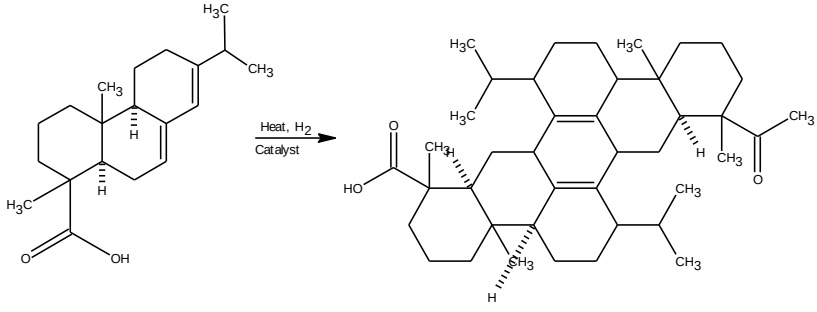
Further reduction can occur causing addition to the last C=C double bond in the molecule. Another approach of stabilization, involves formation of an aromatic ring. This is done by means of disproportionation. Two rosin acids are heated over a catalyst resulting in a redox reaction elimination occur in one of the acids, and addition in the other. (Class 2000).



(1.2)

Polymerization or dimerization is achieved under acidic conditions at high temperatures.

Dimerization occurs as Diels Adler condensation of the double bonds and stabilizes the molecules (Class 2000).



(1.3)

Rosin acid is commonly sold as derivatives. The derivatives serve a variety of purposes in different industrial processes. Sodium and potassium soaps of the resins are used as emulsifiers in rubber production. Calcium and zinc resinate are used in the printing industry in the ink formulations (Class 2000). Copper resinate is formed in coatings containing both copper and rosin, the compound is insoluble in seawater and can therefore damage the effect of the antifouling coating (Redfield et al. 1952).

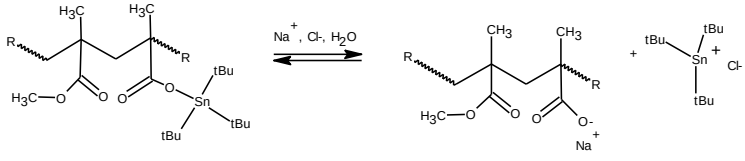
Ester gums is the glycerol esters of rosin acids. They are said to be the most important derivatives of resin acids (Class 2000). They have a wide variety of softening points and hence a wide range of uses. The low solubility in water of ester gum is used to modify the water solubility of the rosin composition of a coating (Redfield et al. 1952).

Chlorinated rubber and cyclic rubber has been used to harden rosin (Redfield et al. 1952), but they are both insoluble in seawater. Rosin alone is not able to form a useful film, and therefore other resins or plasicizers are added to provide coatings of suitable characteristics (Brady and Drisko 2000).

Vinyls are used to provide hydrophobicity in the coating. Vinyl-coatings are generally tough and flexible, and they cure fast even at low temperatures (Brady and Drisko 2000). Vinyl chloride acetate, poly(vinyl butyrate) and poly(vinyl methyl ether) is amongst others reported used in antifouling coatings (Redfield et al. 1952). Vinyl-rosin based antifouling coatings with cuprous oxide as antifouling agent enjoyed a lot of success in the time following World War Two, but they require large amounts of solvents and the VOC content caused a major decrease in popularity, primarily due to legislation (Brady and Drisko 2000).

Chlorinated rubber provides excellent resistance to acid, water, salt and alkaline environment. It can cure in temperature ranges between -35 ^{0}C and 120 ^{0}C, and it is tough and resistant to chemicals in the same manner as vinyls (Brady and Drisko 2000).

Acrylic resins were especially used in connection with TBT. The mechanism of the release of TBT from an acrylic resin is depicted in equation (1.4).



(1.4)

Zinc resinate is the salt of rosin and Zinc. It is used to increase hardness and lower drying time in modern antifouling coatings (Yebra et al. 2004).

Pigments

Pigments are particulate organic or inorganic solids that are insoluble in and unaffected by, the vehicle in which they are included. They can be divided into toxic pigments and non-toxic pigments (Anderson 2000). For non-antifouling uses, the primary cause of pigments is to provide opacity and colour (Yebra et al. 2004). For these applications it is of vital importance that the pigments is insoluble in water, but for antifouling purposes, it is needed that the toxic pigments is water soluble to a low extent. Toxic pigments are described under antifouling agents in section three. In antifouling coatings pigments are also added to provide colour and opacity. As this should not affect the antifouling efficiency over longer periods, it will not be dealt with further here. Other pigments are added as extenders or fillers. The addition of extenders can be varying in nature of origin, but generally they are used to reduce the costs of the coating. Examples of extenders are Barium sulphate, Calcium carbonate, Calcium sulphate and silicate (Yebra 2005).

A characteristic coating parameter is the Pigment Volume Concentration (PVC). It is found by dividing the total volume occupied by pigments, by the total volume of the dry-film (pigment + binder).

$$PVC = \frac{V_{pigment}}{V_{pigment} + V_{binder}} \tag{1.5}$$

The Critical Pigment Volume Concentration (CPVC) is defined as the PVC at which the amount of binder is just sufficient to fill the voids between the pigments. It is evident that the CPVC is dependent on the structure of the pigments.

At PVC values above CPVC, many coating properties are compromised, water permeability and loss of mechanical strength are some. On the other hand blistering is more likely to occur at low PVC's, therefore the optimal pigment content will often lie around (but below) the CPVC (Yebra 2005).

Solvents

The solvents used in coating manufacturing can be divided into true solvents and diluents. True solvents cover the cases where the binders are solubilised in the solvent. As the name implies, solvents are used to solubilise the binder during the coating production. As it evaporates during film formation, the solvent should have no effect on the resulting coating. Usually mixtures of solvents are used to achieve the right combination of viscosity, evaporation rate and solubilising power (Yebra 2005). Solvents commonly used includes: methyl isobutyl ketone and hydrocarbons as toluene and xylene. Some coating systems are not based on a solvent, as the binder material is not solubilised in the liquid phase, the liquid acts as a diluent. This is the case for all water based coatings. The applicability of organic solvents is reduced by legislation. In order to reduce the amount of volatile organic carbon (VOC), water based systems are becoming increasingly popular.

Additives

To improve coating performance and stability, a number of additives can be introduced to the system. Examples of additives include: anti-corrosive, antifoaming, anti settling, anti skinning, can corrosion inhibitors, anti gassing, dispersion aids, driers, modifiers of electrical properties, flash corrosion inhibitors, floating and flooding additives, in-can preservatives, in-film preservatives, insectisidal, optical whiteners, reodorants and UV absorbers (Yebra 2005). These additives should not affect the antifouling coating properties and is therefore not of interest.

Mechanisms of biocide release

The applicability of an antifouling coating relies on 2 parameters, the toxicity of the biocide, and the delivery mechanism of same. The discussion of biocides is saved for section three, but the

mechanism of their release is of equal interest. In general, the biocide delivery mechanism can be divided into three steps. First water penetrates into the coating, this is followed by dissolution of biocide, and the last mechanism is the transport of biocide from the point of dissolution to the surface of the coating (Redfield et al. 1952). Three different ways of controlled biocide release will be described here, the insoluble matrix, soluble matrix and the mechanism of the self polishing copolymer (c.f.Table 1.5). The 2 former are generally applied, whereas the latter is especially concerned with the use of tributyltin as biocide.

Insoluble matrix

Insoluble matrix coatings are also referred to as contact leaching and continuous contact coatings (Yebra et al. 2004), the mechanism relies on the insolubility of the binder/matrix combined with the solubility of the biocidal pigments. The mechanism of biocide release and a graphic illustration of the release over time are shown in Figure 1.10. The dissolution of the pigments creates a porous structure of the coating, because the matrix remains undissolved (Yebra et al. 2004). If the transport of solubilised biocide is taken to be due to diffusion, the flux will depend on the penetration depth in an inversely proportional manner according to ficks law. This means that the flux will decrease rapidly. Initially the flux is very high compared to the efficient level. A large amount of biocide is wasted this way, which not only gives rise to unnecessary pollution, but also reduces service life compared to the max achievable level, based on the initial biocide content of the coating (Redfield et al. 1952). Formerly it was believed that contact between pigments was necessary for the mechanism to work (Redfield et al. 1952). This is the reason for the name “continous contact coatings”. But according to Marson (1969), even pigments fully covered in binder will eventually take up a little water which will cause the surrounding binder to burst, exposing the content of the pigment granule. Therefore the mechanism also works for pigment volume concentrations below the critical level. A consequence of the release mechanism and the subsequent biocide waste of insoluble matrix coating is that the operational life can be determined beforehand, and assigned a specific depth of penetration. Should a higher coating thickness be applied beyond this value, the operational time is unaffected (Redfield et al. 1952). The binder system of insoluble matrix coating is typically based on a high molecular polymer, as they usually are less water soluble. Insoluble vinyl resins, acrylic resins and chlorinated rubbers are generally used (Yebra et al. 2004). The benefits of these coatings are mechanical strength and stability towards oxidation and photo-

degradation (Yebra et al. 2004). The operational time is however below 24 months (Anderson 2000).

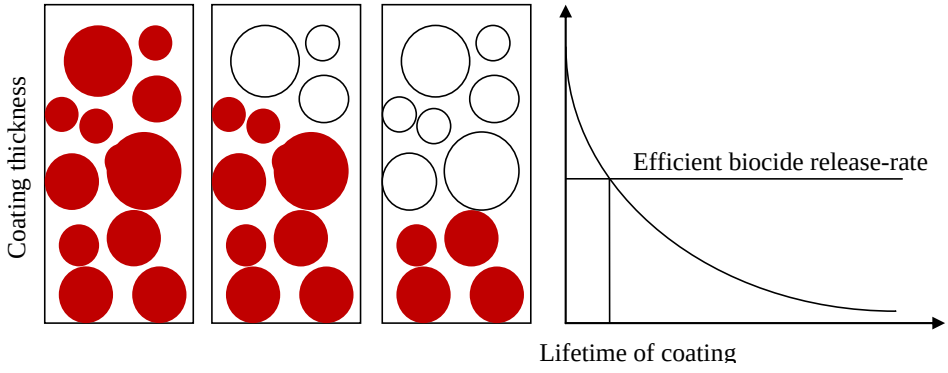


Figure 1.10: Mechanism of biocide release from insoluble matrix systems under dynamic conditions.

Soluble matrix

Soluble matrix antifouling coatings are historically the most used coatings. Modern time developments have modified the conventional binder and mechanism. Nowadays pseudonyms such as controlled depletion, eroding, ablative, polishing, self-polishing and hydration is used alternately to soluble matrix (Anderson 2000). The mechanism of soluble matrix antifoulings is depicted in Figure 1.11. It is based on physically dissolution of rosin into seawater. When in contact with seawater, the pigments will generally dissolute faster than the matrix material. But in contrast to the insoluble matrix coating, the outer boundary of the coating will move, increasing the operational stability. The part of the coating, emptied of pigment is referred to as the leached layer. As the pigments will continue to dissolve faster than the binder material, the leached layer will grow in thickness and eventually cause ineffectiveness of the flux in a manner like the one described for insoluble coatings (Yebra 2004). In general the influence of ship speed depends on the relative rosin content. At low rosin concentrations the velocity does not affect coating performance, and at high concentrations the relationship is exponential (Yebra et al. 2005). Generally the service life of soluble matrix antifoulings is within 36 month (Anderson 2000) high dissolution rate of the matrix is generally connected with good antifouling efficiency (Yebra et al. 2004), therefore relative thick coatings (~500µm) are necessary to obtain antifouling effect over longer periods (Yebra 2005). The major drawbacks of soluble matrix coatings are due to the presence of rosin. First of all rosin is a

brittle material, therefore a high rosin content (high matrix dissolution rate) is connected with low mechanical strength (Yebra 2005).

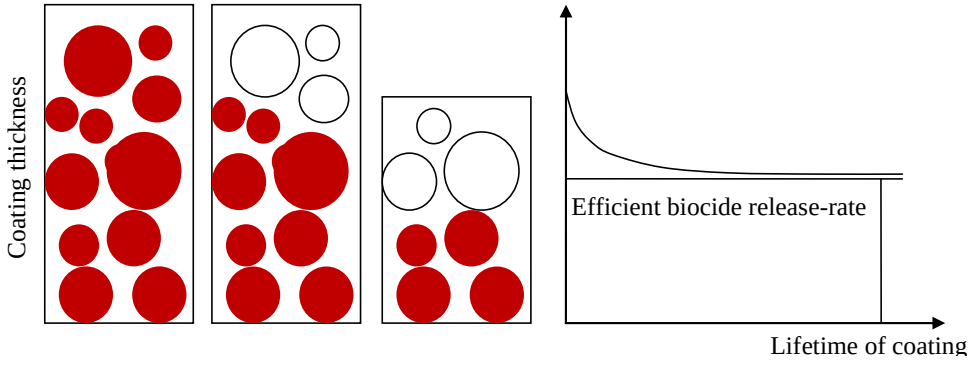


Figure 1.11: Mechanism of biocide release from soluble matrix systems under dynamic conditions.

Self-polishing

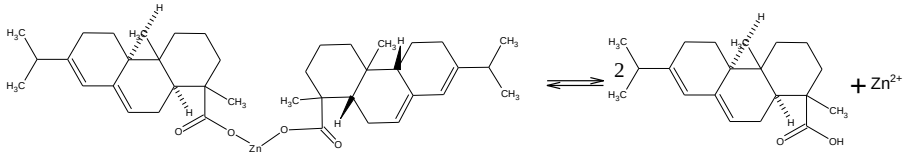
Self-polishing coatings rely on chemical dissolution of the binder material. Whereas the matrix dissolved "physically" in the case of the soluble matrix coatings, the binder material of self-polishing coatings interacts chemically with seawater to become soluble (Yebra 2005). The coating is often based on acrylic polymers, e.g. tributyltin methacrylate methylmethacrylate copolymers. The mode of action relies on hydrolysis of the ester bond between the organotin moiety and the polymer-backbone as seen in (1.4). This unleashes the biocide. Another consequence of the hydrolysis of the ester bond is the resulting carboxylate group in the polymer. This adds to the hydrophilicity and brittleness of the copolymer, and when a suitable amount of ester groups have been hydrolyzed, the seawater can erode the outer layer of the coating and a fresh layer can be exposed. In this manner hydrolysis of the ester bond controls both the release of biocide and the polishing of the coating matrix and the hydrophobic properties of the monomers is a way of controlling polishing rate (Kiil et al. 2001). The coating is added supplementary biocide (Cu_2O), but in general the pigment loading is low (Yebra et al. 2004). The lifetime of a self-polishing copolymer tributyltin coating (SPC-TBT) is directly related to the coating thickness. Applied in sufficient thickness (approximately 400-500 µm) the operational time can be up to 5 years (Yebra et al. 2004). The hydrophobic nature of the copolymer prevents the penetration of water into the coating. The only water penetration occurs in pores left by dissolved pigments. As the pigment content generally is low, the leached layer of an SPC-TBT antifouling coating is very small (Kiil et al. 2001). As the

pigments are not in continuous contact, the dissolution of pigment is also controlled by the polishing rate. Ideally the thickness of the leached layer is therefore constant during polishing. It has been found that the self-polishing effect of the coating is more pronounced at rough spots, this means that the coating will keep a low roughness and consequently low drag resistance. This property is referred to as a self-smoothing effect (Yebra 2005). The polishing mechanism and TBT release rate is remarkably constant. The primary cause of fluctuations is the seawater conditions (Kiil et al. 2001). It is very well understood, and controlled so well that it is possible to design coatings of high and low polishing rate, and consequently produce the proper coating for ships of either low or high activity (Yebra 2005). Another benefit compared to the coatings described above is the antifouling activity at zero velocity (Kiil et al. 2001).

The SPC-TBT coatings are mechanically strong, and resists UV-light as well as atmospheric oxidation, and the drying time is less than for other similar products (Yebra et al. 2004). In general these coatings are preferred with respect to many parameters, only the interaction between biocide and polymer has not been fully replaced by another, environmental benign biocide. Therefore, in practise, the methacrylate copolymer systems alone are not applicable due to the ban of TBT.

As the ban of organotin residues has become effective, the need of a proper substitute has challenged the creativity at coating companies around the world. The commercially available coatings can be divided into two concepts: Controlled Depletion Systems and (Tin-Free) Self-Polishing Copolymers (Yebra et al. 2004). As mentioned above the former is a derivative of soluble matrix coating. The latter is designed to apply the same reaction mechanism utilized by SPC-TBT. The term self-polishing is applied inconsistently. Some are applying the term on acrylic based coatings and others on modified controlled depletion systems. The difficulties with respect to producing a SPC coating with same properties as TBT-SPC, but based on a different biocide, is that the groups attached to the polymer-backbone influences the film properties greatly. The hydrophilicity of the matrix, glass transition temperature shift and swelling is influenced by the groups. This means that even though intentions are good a complete “copy” of SPC-TBT cannot be achieved just by utilization of acrylics. Presently, silylated acrylic backbones are the most popular TBT-SPC mimicking binders, but copper- and zinc- acrylates are also known of (Yebra et al. 2004).

Zinc resinate is widely applied as binder material in commercial self-polishing coatings. The mechanism of zinc resinate is probably ion exchange (Yebra et al. 2005). The mechanism is illustrated in equation (1.6):



(1.6)

Mathematical modelling

Mathematical modelling of antifouling coatings is described for the different types of coatings. The first model described, is based on an insoluble matrix coating. Marson (1969) estimates the effect of pigment volume concentration, temperature and fluid velocity. Based on assumptions of equal size of spherical Cu_2O particles he created a model that could be solved analytically. The resulting model showed fairly good correlation with experimental data. Based on the model Marson suggested that PVC should be kept high to achieve maximal leaching rates. De la Court and De Vries (1973) concludes that the chloride and copper content of seawater is influencing leaching rates greatly. Temperature was found of no influence. The model created is similar to that of Marson; only the shape of pigments and distribution in the coating is added to the model (Kiil et al. 2002a). Monaghan et al. (1978) aimed to minimize the needed amount of testing to predict lifetime of insoluble matrices. The coating type that was described was an insoluble matrix, leaching organotin as biocide (see Kiil et al. 2002a). Insoluble matrix coatings was until recently the most frequent antifouling coating subjected to mathematical modelling (Kiil et al. 2002a). However, soluble matrix coatings have not been a subject of equal interest when it comes to modelling (Kiil et al. 2002a). Only a simple model of TBT-SPC coatings is described by Somaskharan and Subramanian (1980), until Kiil et al. took it upon themselves to provide a detailed model of the mechanisms involved in the antifouling activity of SPC-TBT.

The model described by Kiil et al. (2001) is based on practical analysis of 5 coatings produced from a simplified formula (not containing additives and extenders). The binder material of the coatings is tribytyltin methacrylate/mehyl methacrylate, and butyl methacrylate is used as copolymer/retarder. The pigments are made up of Cu_2O particles and the solvent is xylene.
The model is involving the hydrolysis of TBT and the erosion of polymer, the dissolution of pigments and mass transport resistance. Of chemical species, H^+, OH^-, Na^+, Cl^-, HCO_3^-, $CO_3^{2-}$from the marine environment are taken into account. $CuCl_2^-$, $CuCl_3^{2-}$ and Cu_2O are considered from the pigments and the three species concerned with the hydrolysis of the TBT methacrylate are also

included. Very good match with experimental data is reported between polishing rate and leached layer thickness for all the coatings containing retarder in small amounts. Without retarder, the polishing rate is very high and the leached layer is absent. The retarder determines the lag time (the time before polishing is initiated) and addition of retarder is causing longer lag times. The importance of Cu_2O on the polishing mechanism is described. No polishing is reported for coating free of pigment. As a variable in the model, Kiil et al. introduces X_{max}. The value of this parameter corresponds to the ratio of TBT groups hydrolyzed in the polymer before the polymer becomes soluble and removed in seawater at given values of temperature and sailing speed (Kiil et al. 2001)

In a parameter study, seawater-, sailing and coating- parameters effect on polishing rate and thickness of the leached layer is tested using the model. It is reported that the pH of the seawater influences both parameters equally. They decrease with increasing pH in the region between 7,6 and 8,6 (Kiil et al. 2002b). The sodium chloride concentration has little effect on polishing rate unless the salt concentrations are very low, like the values for fresh water. The leached layers thickness is also influenced by salinity. It is changed a factor of three if the sodium chloride concentration varies from 0.4 to 0.8 mol/liter (Kiil et al. 2002b).

Cu_2O particle size does not influence the rate of dissolution of copper species directly. This is due to the steep copper profile at the interface between the dry film and the leached layer. The conversion rate of polymer in the leached layer is, however, dependent on the particle size distribution of the copper particles. This is because the water penetrates into the pores left by the leached particles. Kiil et al. finds that at small particle sizes (<3 µm) the polishing rate increases with decreasing particle sizes, as the surface volume of the leached layer is increased. At higher diameters, the tendency is significantly less pronounced. The thickness of the leached layer is inversely proportional to the polishing rate (Kiil et al. 2002b). The pigment volume concentration is expected to affect the coating properties, because the pigment content again determines the active surface area of the leached layer. Kiil et al. finds that for PVC varying from 30 to 48% (of CPVC) the polishing rate increases almost linearly from 12 to 16 µm/month. Accordingly the thickness of the leached layer decreases in the same region (Kiil et al. 2002b).

Dynamic simulations are also described in a paper of Kiil and co-workers. It is found that stable conditions for the coating described by the model, is seldom reached due to slow responses to changes in parameters such as temperature and speed. Steady state conditions are reported to develop faster for increases rather than decreases of these parameters (Kiil et al. 2002c).

Unfortunate combinations of temperature and speed will cause leached layers of great thicknesses (Kiil et al. 2002c).

Rosin based (SP) coating modelling

A rosin-based coating has also been described by mathematical modelling (Yebra et al. 2006). In a thorough work the forecast of the model is compared to experimental data obtained. The conclusion is used to determine the validity of the underlying assumptions. The modified assumptions are compared to those used by Kiil et al. to model TBT-SPC:

Table 1.6: Assumptions believed to describe rosin based SP systems and SPC-TBT respectively. From (Yebra 2005).

	SP/Rosin	SPC-TBT
Copper leaching	• Significant seawater penetration beyond pigment front • Saturation of Cu(I)-species at the pigment front • Purely diffusion-controlled process	• Negligible seawater penetration beyond pigment front • Kinetics of Ferry and Carrit (1946) apply • Chemical reaction and diffusion determine the leaching rate
Pore morphology	• The pores grow in size with exposure time • Low tortuosity factors • Low pore surface area per total paint volume • Few "bottleneck" structures	• Poresize remains largely unchanged • Higher tortuosity-factors • Large pore surfaces • "bottlenecks" formed
Binder reation	• High mass loss rates • A priori, the exposed binder surface can be renewed during exposure • Release of Cu^{2+} might influence binder reaction rate • Reaction seems to be progressively hindered as the conversion increases • Difficulty in reaching a high conversion value	• Low mass loss rates • The exposed binder surface is not renewed during exposure • Only the pH and Cl^- concentration have been reported to affect the hydrolysis rate • High conversion values may be attained (TBT groups beyond the porewalls can be attacked by seawater)

Furthermore, the following tendencies for the rosin based antifouling coating are listed (Yebra et al. 2006):

- The erodability of the paint system is influenced by the insoluble paint constituents.
- The leaching rate of copper is strongly dependent on the hydrophobicity of the co-binder.
- Erosion rate of systems based on a large amount of seawater soluble pigment is dependent on seawater solubility even though no leached layer arises.

- In the other end of the scale, systems containing small amounts of seawater soluble pigments have a linear relationship between mechanical strength of the leached layer and content of seawater soluble pigments.
- Small particle sizes of the insoluble pigments create paints with greater erosion resistance.
- Tortuosity is increased with higher pigment volume concentrations and smaller particle sizes.
- Threshold value of PVC of insoluble pigments, that should be exceeded to obtain highly stabilized leached layer of low erosion rates. (Yebra et al. 2006)

It is concluded that the leaching rate can be controlled by combining hydrophobic resins, and insoluble and soluble pigments. But to achieve this within a reasonable timeframe, knowledge of the novel coating parameters (X_{max} etc.) is required; these can best be attained with the use of the computer based models and accelerated tests (Yebra et al. 2006).

Other antifouling approaches

Fouling Release coatings differ greatly from the other commercially available antifouling coatings. The principle is as old as that of Self-Polishing Copolymers, but the effectiveness and economic benefits of SPC dominated the market until the 1990s where the ban of TBT started (Yebra 2004). The conclusions of the studies done to elucidate adhesion of foulers to coatings has been summarised in Yebra et al. (2004). The main properties a coating should have to resist fouling due to its physical properties are repeated below:

- Flexible, linear backbone
- A sufficient number of surface-active groups that can move to the surface freely
- Low elastic modulus
- Smooth surface at molecular level
- High molecular mobility in the backbone and surface active side-chains
- A thickness which can control the fracture mechanics of the interface
- Molecules which combine all of the above factors and are physically and chemically stable for long periods in the marine environment. (Yebra et al. 2004)
- Low surface energy

Fluoropolymers has been intensively investigated because of their extremely low surface energies, but they have fallen short compared to silicones as they provide mechanical stiffness and low

critical stress. Therefore the fouling accumulated on Teflon is not released under sufficiently low stress (Yebra et al. 2004). Silicones in high thickness is due to relatively low surface energy, micro roughness and glass transition temperature, combined with very low elastic modulus, better suited as a fouling release coating, even though the surface energy is higher than that of fluoropolymers. Poly(dimethylsiloxane) coatings are the most commonly used today (Yebra et al. 2004). The release of fouling organisms from a poly(dimethylsiloxane) based coating is slower compared to fluoropolymers, but cost less energy. The most coatings, commercially available, also contain fluid additives. These short-chain silicones are expected to leach from the coating creating even more flexible surface, thus adding to the fouling release efficiency. Depletion of the short chained additives makes the coating crack. Therefore it is the upper limit of service life. Efficient fouling release only occurs at high velocities, creating a limit of applicability of the coatings, regarding operational frequency and velocity. Other drawbacks are price, difficulties combined with repair, combination of silicone technology in coating systems, and the unknown environmental consequence of the leaching silicones (Yebra et al. 2004).

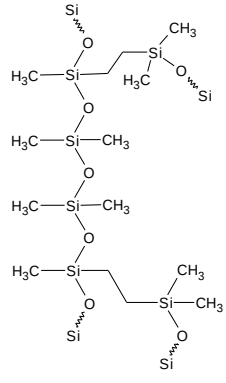


Figure 1.12: Schematic illustration of cross-linked poly(dimethylsiloxane)

Combination of antifouling and fouling release technologies has been investigated, but it is obvious that the delicate nature of the fouling release coating is limiting the possibilities, making development of a biocidal controlled release system based on siloxane technology a difficult task (Yebra et al. 2004).

Alternatives to the conventional biocide based (and recently also fouling release coatings) that are dominating the market, is primarily concerned with production of electrical current in the coating. Initially, it was with the intention to produce toxic species (chlorine) electrolytically. The

resistance of the coating and thus the very large voltage drop, promotion of coating ageing, and increased corrosion was the primary drawbacks of these coatings. Other species produced electrolytically involves: ozone, copper ions, hydrogen peroxide, platinum complexes, bromine, and ammonia (Yebra et al. 2004). Electrolytic production of most species requires relatively large voltages. Direct electrically inhibition of fouling based on lower voltages has also been studied intensively, oscillating potentials is improving performance for these concepts. The conductivity of the coating is of great relevance for these systems (Yebra et al. 2004).

Antifouling effect of acoustic radiation has been proved (Yebra 2005). The radiation can either be applied by external vibration, or the use of piezo electric materials. The former can arise from use of loudspeaker-like technology and the latter by adding piezoelectric materials to the coating.

Magnetic fields has been tested for antifouling efficiency, the tests showed possitive result for some fouling species. Ultraviolet radiation is used to sterilize seawater, but it is too costly to apply on large surfaces. Radioactive coatings are also described in the literature. ^{204}Th, ^{95}Tc and ^{99}Tc is mentioned. Generally the applicability is limited either due to inefficiency, or unsafe handling (Yebra et al. 2004).

Enzymatic antifouling has also been described in several occasions. Chapter two will deal with this approach to fouling control.

Applying a coating

When applying a coating, the parameters of highest importance are the film forming properties and the coating thickness. The coating thickness is especially important for antifouling coatings, as the coating should contain biocide in an amount sufficient for the entire operational time of the coating. In principle paint can be applied by any means thought of. But with respect to coating thickness, one should choose a method that can provide good control of the film thickness during application. The common methods of applications are by means of a brush, a roller and conventional or airless spray (Class 2000). The brush and roller are easy to use and they are probably the most common in applications on yachts, as the surfaces are relatively small and the spray methods are more expensive. Of these two methods, the brush provides the best film thickness-control (Class 2000).

Conventional spray is commonly applied in combination with low-viscosity paints (Class 2000). Paint and air is combined in the process of making aerosols. The pressure of air is typically in the range of 0.28 to 0.56MPa (Class 2000). Airless spray is produced by forcing the paint

(without air) through a nozzle at pressures in the range of 20MPa (Class 2000). The high pressures are related to some health risks and such a system should exclusively be handled by trained personnel.

The number of layers applied to the ships hulls differs between recreational and industrial vessels. For yachts, the antifouling coating is usually applied on top of a two-component sealer, and any metallic parts of the bottom are usually coated with a copper-free paint to avoid corrosion.

Generally, the hulls of industrial ships are coated with 3 layers, a primer, an undercoat and the coating providing antifouling effect. The primer is supposed to seal the substratum and provide anticorrosive effect, and the tiecoat is used to promote adhesion of the topcoat to the substratum. Finally the topcoat is used to achieve antifouling effect (Yebra 2005). Apart from the preeceding 4 lines, this paper exclusively deals with the antifouling coating.

Ship owner's requirements

The needs of an antifouling coating differ according to the nature of the sailing. Pleasure crafts are usually above waters for a part of the year. For the northern regions, the temperature means that the use of a yacht is limited to only approximately half a year. Therefore the demands from yacht owners are expected to be cost related. If the coatings efficiency exceeds 8 month, the operational requirements will be fulfilled. From this point forward, the interest is primarily economic. Issues as the environmental consequences will for some yacht owners also have a saying, but in the same spirit conservative sailing enthusiasts can be thought to wish to use what has been working for the last many years (TBT).

Industrial ships are preferably in the sea constantly. The operators lose money, when the ships are docking. Not only because of the expenditure connected with renovation, but lost income due to inactivated ships are the major concerns (cf. section one).Therefore the interests of industrial ship owners are concerned with operational stability primarily. Of course the ship owner would always want the cheapest coating, but the coating expenditure can be minimized when the costs is compared to higher dry docking intervals, and significantly increased fuel consumption.

Antifouling agents

This section deals with the agents used in antifouling coatings to provide the fouling inhibiting effect. Biocidal action in general will first be covered. This is followed by a discussion of the conventional biocides. Supposed environmentally friendly antifouling agents will follow (enzymatic

induced antifouling is described in chapter two). Last in this section, a brief description of the most important legislation on the area is explained.

Biocidal effects

Biocides's effects on bacteria can be divided into bacteriostatic and bacteriocidal effects. The former covers reversible injury, typically caused by metabolic interactions. It is reversed following removal or neutralization of the biocide. Bacteriosidal interactions cause irreversible damage to cellular structure or function (Denyer 1995). Interaction between organisms and biocide follows this general chain of events: 1, Uptake of biocide by cell; 2, partitioning/passage of biocide to target; 3, concentration of biocide at target; 4, damage to target. The uptake of biocides may account for a great variability in the effect of the given biocide. Gram positive cells and gram negative cells differ on the lipopolysaccharide layer on the outer membrane of the gram negative cells. Thus the uptake of one biocide by a gram positive cell may be larger than for a gram negative cell and vice versa (Denyer 1995).

Table 1.7 shows a summary of the reaction targets and consequences for the impact of some common biocides on bacteria.

Table 1.7: Interactions of common biocides on bacteria. Modified from (Denyer 1995).

Target region	Damaging event	Consequence	Biocide	Reaction	Inactivation/ neutralizing
Cell wall	Structural/functional changes; release of wall components; initiation of autolysis	Abnormal morphology and construction; non-specifik increase in cell permeability; lysis	Phenol	Partitioning into phospholipids bilayer	Dilution
			Hypochlorite	Oxidation of thiol groups; Halogenation	
			Formaldehyde	General alkylation reactions	Glycine
			Mercurials	Oxidation of thiol groups	Cysteine/ thioglycollate
			Cetytrimethylammonium bromide		
			Aliphatic alcohols	Solvation of phospholipids	Dilution
			2-phenylethanol	Solvation of phospholipids	Dilution
			EDTA		
			Sodium dodecyl sulphate	Membrane-protein solubilization	
			4-Aminobenzoic acid	Partitioning into phospholipids bilayer	

Cytoplasmic membrane	Loss of structural organization and integrity	Progressive leakage of intracellular material; initiation of autolysis	Quaternary ammonium compounds	Electrostatic interaction with phospholipids	Lecithin
			Phenol	Partition into phospholipids bilayer	Dilution
			4-ethylphenol	Partition into phospholipids bilayer	Dilution
			Tetrachlorosalicylanilide	Oxidation of thiol groups	
			Fentichlor		
			Sodium dodecyl sulphate	Membrane-protein solubilization	
			Ethanol	Solvation of phospholipids	Dilution
			Chlorhexidine	Oxidation of thiol groups	
			Polymeric biguanides and alexidine	Electrostatic interaction with phospholipids	Lecithin
			2-phenylethanol	Solvation of phospholipids	Dilution
			N-dodecyldiethanol amine		
			Triclosan		
			Polyethoxyalkylphenols		
	Selective increase in permeability to protons and other ions	Dissipation of proton motive force; uncoupling of oxidative phophorylation; inhibition of active transport; loss of metabolic pools	Lipophilic weak acids	Partition into phospholipids bilayer	
			Parabens		
			Alkylphenols	Partition into phospholipids bilayer	Dilution
			Chlorocresol		
			2-phenoxyethanol	Solvation of phospholipids	Dilution
			Fentichlor		
			TCS		
			1-dodecylpiperidine N-oxide		
	Inhibition of membrane-bound enzymes	Inhibition of respiration and energy transfer; inhibition of ATP synthesis; inhibition of substrate oxidation; inhibition of transport processes	Chlorhexidine	Oxidation of thiol groups	
			2-phenoxyethanol	Solvation of phospholipids	Dilution
			Azide		
			Bronopol		Cysteine/ thioglycollate
			Cetytrimethylammonium bromide		
			Isothiazolones	Oxidation of thiol groups	Cysteine/ thioglycollate
			Iodoacetate		
			Organomercurials	Oxidation of thiol groups	Cysteine/ thioglycollate
			Heavy metal salts	Oxidation of thiol	

			(silver, copper, mercury)	groups	
Cytoplasm	Inhibition of cytoplasmic enzymes; interaction with functional biomolecules	Inhibition of catabolic and anabolic processes	Formaldehyde	General alkylation reaction	Glycine
			Acridine dyes		
			Oxidizing agents (Hydrogen peroxide peracetic acid)	Free radical oxidation	
			Parabens		
			Chloroacetamide	Oxidation of thiol groups	
	Coagulation and precipation of cytoplasmic constituents	Denaturation of enzymes; destruction of biomolecules	Chlorhexidine (biguanides)	Oxidation of thiol groups	
			Quarternary ammonium compounds	Electrostatic interaction with phospholipids	
			Some phenolics	Partition into phospholipids bilayer	
			Some heavy metals	Oxidation of thiol groups	

The biocides listed in Table 1.7 are not all suitable as antifouling agents. The table is rather meant as an overview of the mechanisms and reactions involved in biocidal interaction with bacteria. And may be used in inspiration to potential enzyme based indirect (cf. chapter two) antifouling products. The following paragraph will deal with the metallic species utilized as "primary" biocides. Afterwards the common organic co-biocides will be described

Metal-based biocides

In general commercial antifouling coatings are based on the biocidal activity of one or more metallic oxides, cuprous oxide and cuprous thiocyanate being the most popular (Voulvoulis et al. 1999). Cuprous oxide is by far the most used "primary" biocide, and the major alternatives are also copper-based species. Thiocyanate and metallic copper is mentioned in the literature (Voulvoulis et al. 1999). Cuprous oxide is solubilised in seawater according to the following mechanisms:

$$\tfrac{1}{2}Cu_2O(s) + H^+ + 2Cl^- \rightleftarrows CuCl_2^-(aq) + \tfrac{1}{2}H_2O \quad (1.7)$$

$$CuCl_2^-(aq) + Cl^- \rightleftarrows CuCl_3^{2-}(aq) \quad (1.8)$$

Reaction (1.7) is reversible and slow. Reaction (1.8) occurs instantly and it can be considered at equilibrium at all times (Kiil et al. 2001). $CuCl_3^{2-}$ will be oxidized by solubilised oxygen, resulting in copper(II)ions, which are the major biocidal species when Cu_2O is used in antifouling coatings (Yebra 2005). The global release of copper due to antifouling is 3,000 tonnes per year, and the

natural source of copper to the aquatic environment is of 250,000 tonnes per year (Yebra 2005). Furthermore copper is not lipophilic and has therefore a low tendency towards bioaccumulation. Due to low water solubility, fast precipitation decreases the toxicity (Yebra 2005). The most toxic compound, of the copper species, is the hexaaquacopper(II) ion, because of the highest bioavailability (Voulvoulis et al. 1999). Speciation between the copper compounds is determined by pH, salinity an organic matter (Voulvoulis et al. 1999). The antifouling activity of copper is primarily against organisms such as barnacles, tube worms and the majority of algal foulers (Voulvoulis et al. 1999). The sensitivity towards copper of the marine animals is decreasing in the order: microorganisms > invertebrates > fish > bivalves > macrophytes.

Insoluble salts of the copper(II)ion, such as carbonate and hydroxide decreases the availability and thus toxicity of copper species (Voulvoulis et al. 1999). If there are stronger ligands than water present, the hexaaqua ion will be converted. Some micro-organisms are believed to use this by synthesizing chelators in response to increased copper levels, to decrease the toxicity (Voulvoulis et al. 1999). Organic ligands, such as the citrate-ion (Redfield et al. 1952) will cause the copper ions water solubility to increase significantly and as a consequence to be biologically inert (Voulvoulis et al. 1999), other organic species, utilized as co-biocides form lipophilic complexes with copper, increasing its bioavailability and thus toxicity. One such biocide is thiocarbamate (Yebra 2005). The toxicity of copper is significantly lower, compared to the organotin species. However the different complexes will result in different fates for the species. The discussion of the environmental consequence of the utilization of copper in antifouling coatings is therefore ongoing. Several researchers claim to have found evidence of the impact on non-target species (Evans et al. 2000), and the increasing use of copper in antifouling coatings has been correlated with an increase of copper found in oysters (Voulvoulis et al. 1999). High copper concentrations are believed to cause deleterious sub lethal effects to invertebrates and lethal effects in early life stages (Voulvoulis et al. 1999). Other researchers aim at the low bioavailability to claim an acceptable environmental profile of the compound (Yebra 2005), more than 99% of the total copper content of the oceans is believed to be strongly bound by organic ligands (Voulvoulis et al. 1999).

The antifouling effect of other pigments in antifouling coatings, such as zinc oxide, titanium dioxide and iron(II)oxide still remains to be investigated as thoroughly as those of copper (Yebra 2005). The effect of zinc oxide is reported to be physically modification of the coating surface. More than one commercialized antifouling coating is based entirely on this oxide to provide

antifouling effect. The coatings are, amongst others, aimed at the market in Sweden where the environment is less tolerant. A study on environmental consequences of novel antifouling coatings includes at least one of these coatings comprising only zinc oxide as fouling inhibiting substance. The researchers conclude, based on a study of toxicity on two species of red algae and one crustacean, that the coating contain "toxic substances" (Karlsson and Eklund 2004).

There are two major mechanisms of toxicity of metallic ions, overload and membrane partitioning. Overload occurs when the concentration of metal ions is high. Most metal ions are essential in small amounts. Therefore organisms have a mechanism to transport them into the cells. This mechanism cannot be shut off. Therefore high concentrations in the water will result in overload. Overload of metal ions in the cells will disrupt the metabolism and ultimately cause death of the cells (Rittschof 2001). The other mechanism is uncoupling of oxidative phosphorylation and electron transport. Lipophilic metal-compounds, such as TBT, can penetrate cell membranes. In the membranes they interfere with essential membrane functions, one of these is the electron transport (Rittschof 2001).

Co-biocides

The organic biocidal compounds used conventionally as co-biocides in commercial antifouling coatings, can be divided into metallic and non-metallic. A list of the more common is presented by Voulvoulis et al. (1999). The list is presented in Table 1.8.

Other molecules reported used is copper pyrithione, isothialozone, benzmethylamide, fluorofolpet, polyphase, pyridine-triphenylborane, TCMS and tolylfluanid (Yebra 2005).

Table 1.8: The list of the most common co-biocides used in antifouling coatings. From: Voulvoulis et al. (1999).

Name	Mechanism
Chlorothalonil 2,4,5,6-tetrachloroisophthalonitrile	Fungicide
Dichlofluanid N'-dimethyl-N-phenylsulphamide	Fungicide
Diuron 3-(3,4-dichlorophenyl)-1,1-dimethylurea	Herbicide Inhibition of photosynthesis Carcinogenic Defromed growth in fish larvae
Irgarol 1051 2-methylthio-4-t-butylamino-6cyclopropylamino-s-triazine	Herbicide Inhibition of photosynthesis
Zinc pyrithione [di2-mercaptopyridine-1-oxide] zinc	Bactericide, fungicide Inhibition of cell growth in mammals Paralysis in rabbits mutagenic potential
Thiram Bis(dimethylthiocarbamoyl)disulphide	Fungicide
Ziram Zinc bis(dimethyl thiocarbamate)	Fungicide
Maneb Manganese ethylene bisdithiocarbamate	Fungicide
Zineb Zinc ethylene bisdithiocarbamate	Fungicide
Kathon 5287 4,5-dichloro-2-n-octyl-4-isothiazolin-3-one	
TCMTB 2-(thiocyanomethylthio)benzothiazole	Fungicide
TCMS pyridine 2,3,5,6-tetrachloro-4-(methylsulphonyl)pyridine	

Irgarol 1051

Irgarol 1051 is one of the better investigated of the compounds listed above. It is highly efficient against algae (Voulvoulis et al. 1999), because its mode of action is to interfere with the photosynthetic electron capture transport in chloroplast (Voulvoulis et al. 1999). It is likely to be found in relatively high concentrations in both the water column and in the sediment and effects of impact on non-target organisms are reported for concentrations of 50ng/L (Yebra 2005). It is reported stable under natural conditions of seawater (van Wezel and van Vlaardingen 2004), and the degradation in the water column and in sediment is slow. The primary sink of irgarol is expected to be photodegradation, but also biodegradation by white rot fungi, and mercuric chloride catalyzed hydrolysis occur. All degradation processes create the major metabolite; 2-methylthio-4-tert-butylamino-6-amino-s-triazine (GS26575), which is also toxic (Voulvoulis et al. 1999). The toxicity is within the range of that of the parent-compound (Okamura et al. 2000). There are opposing results when it comes to the stability of GS26575 in seawater (Konstantinou and Albanis 2004).

Diuron

Diuron is one of the most used co-biocides. It is the biocide which is found in the highest concentrations in seawater (Konstantinou and Albanis 2004). The high load of diuron found is also related to its use in weed control in non-agricultural applications (Konstantinou and Albanis 2004). The toxic mechanism is inhibition of photosynthesis in a mechanism similar to that of irgarol (Konstantinou and Albanis 2004). In seawater of moderate pH, diuron is not hydrolyzed to any significant extent (Yebra 2005). However it is biodegradable under aerobic and anaerobic conditions (Yebra 2005). Its metabolites is: 1-(3,4-dichlorophenyl)-3,1-dimethylurea (CPDU); 1-(3,4-dichlorophenyl)-3-methylurea (DCPMU); 1-(3,4-dichlorophenyl)urea (DCOU) and demethyldiuron. Diuron is not expected to bio accumulate significantly (Voulvoulis et al. 1999) and studies in UK have led to no findings of Diuron (Voulvoulis et al. 2000). Diuron contamination is, however reported for several European countries, (Evans et al. 2000).

Diuron, Irgarol, Kathon and Chlorothalonil has been investigated for degradability, they are reported to be almost exclusively degraded biologically (Voulvoulis et al. 1999).

Sea-nine 211

Sea-nine 211 is biological active against a wide spectrum of bacteria, diatoms, fungi and algae (Yebra et al. 2004). It binds strongly to the sediment which reduces its bioavailability greatly. Biodegradation is fast which probably can be related to the broad activity. Within days it should be decreased to non-toxic levels. Also its photo degradation is fast compared to the compounds mentioned above. As no bioaccumulation is expected, Sea nine is accepted as a compound with a more environmentally friendly profile. However contamination of the biocide in waters where it is not used has been reported (Yebra et al 2004).

Dithiocarbamates

Dithiocarbamates are known to have synergistic effect with copper(II)ions. Several studies report at least an order of magnitude increase in potency in combination with copper(II) (Voulvoulis et al. 1999).

Dichlofluanid

Dichlofluanid is unstable in alkaline waters. It can not be measured at pH values of 9 as it decomposes to fast (van Wezel and van Vlaardingen 2004). Its primary metabolite is dimethylaminosulphanilide (DMSA), which is toxic also, therefore, the environmental profile of dichlofluanid should involve the nature of DMSA (van Wezel and van Vlaardingen 2004). Another

less important metabolite is dichlorofluormethane. It is proposed that the primary degradation of dichlofluanid is over DMSA, both metabolites can degrade further to give aniline (Thomas et al. 2003).

Chlorothalonil

Chlorothalonil is decomposing due to photolysis and biodegradation (Konstantinou and Albanis 2004), though biodegradation occur primarily in soil. There are several degradation products, one of them, 4-hydroxy-2,5,6-trichloroisophtalonitrile is also toxic and very stable, resulting in potential accumulation (van Wezel and van Vlaardingen 2004).

Ziram

Ziram decomposes fast due to hydrolysis, the major product is CS_2 (van Wezel and van Vlaardingen 2004). As CS_2 is not toxic, the toxicity of ziram, decreases fast, thus improving its environmental profile in comparison with other conventionally co-biocides (van Wezel and van Vlaardingen 2004).

TCMTB

TCMTB decreases relatively fast, aerobic metabolism and photolysis is the cause of degradation. In both cases, the metabolite is 2-mercaptobenzothiazole (MBT). MBT is also toxic and therefore the degradation does not remove toxicity (van Wezel and van Vlaardingen 2004).

Zinc and copper pyrithione

The metal (zinc and copper) salts of pyrithione are the most interesting of the co-biocides that are metal based. They are functional as algaecides, fungicides and bactericides (Yebra et al. 2004). The cause of toxicity in the compounds is reported to be the N-hydroxythioamid group (Doose et al. 2004). If degradation is believed to remove the pyrithione, care has to be taken that this particular group breaks down (Doose et al. 2004). The photodegradable characteristic makes pyrithione favourable from an environmental point of view. Although it should be considered that the risk of accumulation is increased in deep waters or where UV-light from the sun is otherwise kept out (Yebra et al. 2004). The benefits of the salts depend on the metallic cat-ion. Copper pyrithione has shorter half-live and is more efficient against softer foulers, and the harder zinc pyrithione is more potent when it comes to hard foulers (Yebra et al. 2004).

Environmentally friendly approaches

The quest for environmentally friendly antifouling agents can be looked upon from different angles. Some scientists are searching for natural occurring compounds that have shown antifouling properties for marine species. Others are searching amongst known short-lived or benign compounds, others are focussing on the potentials of inhibiting quorum sensing and yet others are concentrating on the potentials of enzymes. This paragraph will deal with the three former, enzymes as antifouling agents will be described in chapter two.

Natural antifouling agents

The observation that several sea living organisms do not foul, has led to the hypothesis that the organisms in question secrete antifouling substances. These substances are in some cases mucus, but in several known cases, the substance expected to provide the antifouling activity is an organic molecule (Rittschof 2001). Even though fouling is a surface phenomenon, and antifouling agents therefore is expected to be related to the surface of the organisms, the research on the area is primarily concerned with extracts of the full body of the organisms (Rittschof 2001). Several researchers have investigated the extracts of a variety of marine sponges, corals, algae and bacteria. It is difficult to provide an overview of the research on the subject. Primarily because of the many species and extracts tested, but also because some researchers refer to the solvent in which they extract rather than the expected antifouling compound (Abarzua and Jakubowski 1995). Generally the families, steroids, terpenes, phenolics, brominated hydrocarbons, brominated tyrosine derivatives, and saponins have been identified as natural antifoulants. Reviews of natural antifouling have been listing the compounds extracted through time. Table 1.9 shows a reduced list as presented by Rittschof (2001).

Due to their widespread existence in nature, it is not surprising that a large amount of the extracts tested is derivatives of terpenes. Diterpenes (terpenes composed of four isoprene units) is described very often (Clare et al. 1999), (Hellio et al. 2001); (Okino et al. 1996). But also sesquiterpenes (terpenes comprising three isoprene units) (König and Wright 1997); (Sera et al. 1999) is well represented, and terpenoids (oxidised or chemically rearranged terpenes) (Clare et al. 1992) and other chemically modified terpenes (Clark et al. 2000); (Anthoni et al. 1991). Steroids (also in distant relation to terpenes) (Tomono et al. 1999) has also been reported as natural antifoulers. The effects on barnacles of four steroids found by Tomono et al. (1999) were lethal in

high concentrations. The steroid glycosides extracted by Palagiano et al. (1996) showed inhibition without causing death.

Table 1.9: Natural products antifoulers tested from 1995 to 2001. From: (Rittschof 2001).

Extract/parent compound	Source
Diterpene	Sponge
$Ca(OH)_2$	Concrete
Nitrogen heterocycle	Sponge
Terpenoids	Sponge
Sesquiterpene	Nudibranch
Cyanoformamide	Sponge
Bromotyrosine	Sponge
Oroidin	Sponge
Steroid	Sponge
Phlortannins	Algae
Polyacetylene	Sponge
Bromophenol	Polychaete
Lentil lectin	Plants
Protein complex (SIPC)	Barnacle
Lumichrome	Ascidian
Homoserine Lactone	Algae
Cardenolide	Insects/plants

Quorum sensing inhibitors

A group of natural antifouling substances is heavily investigated as they are expected to be completely harmless to the fouling species as well as non-target organisms. It was described in section one that fouling species are known to "communicate" by quorum sensing. If the micro-organisms are prevented from quorum sensing, the tendency to foul diminishes (Smit 2004). Several studies on both natural and synthetic organic molecules indicate the reversible anti settlement effect of inhibiting quorum sensing (Pereria et al. 2002). As mentioned, gram negative bacteria use acylated homoserine lactones (AHL) during quorum sensing. Therefore molecules that structurally resemble AHL can potentially inhibit quorum sensing, in a manner similar to competitive inhibition in enzyme technology. Furanones and lactones align the spatial structure of AHL. Furanones especially, are the compounds making up the majority of investigations focused on the reversible inhibition of fouling. Figure 1.13 shows furan and acylated homoserine lactone.

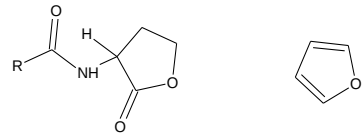


Figure 1.13. Left: Acylated Homoserine Lactone (AHL). Right: Furan.

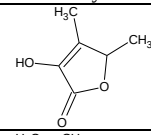
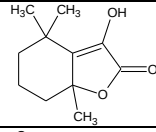
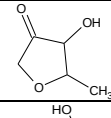
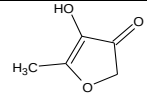
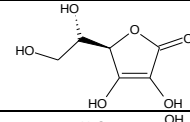
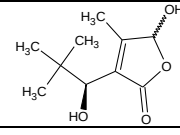
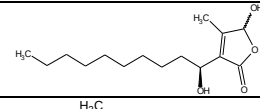
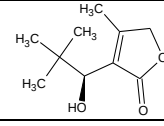
Marinelli et al. (2004) describes the inhibitive effect of both synthesized and commercially available furans. From a coating producer's point of view, the commercial availability is essential. Therefore only some of the commercially available furans together with the most potent inhibitors that was described by Marinelli et al. are listed in Table 1.10.

The effect of AHL inhibitors can be difficult to determine due to the non-linear relationship between concentration and effect (Marinelli et al. 2004). Some of the furanones included in the study of Martinelli et al. (2004) work as agonist of the quorum sensing transmitter at low concentrations, and as antagonist at high concentrations. Therefore exact determinations of the potential of a furan as antifouling agent need plenty of studies of varying concentrations (Marinelli et al. 2004).

Ascorbic acid (Novak and Fratamico 2004) is included in Table 1.9 because it is a stereoisomer of erythorbic acid. Erythorbic acid is interesting because it is the product of the oxidation of D-gluconolactone by gluconolactone oxidase, a reaction that also produces hydrogen peroxide (see chapter two). Ascorbic acid is shown to inhibit autoinducer 2 in a foodborne pathogen (Novak and Fratamico 2004). In a literature search, no paper were found, describing potential quorum sensing inhibition of D-gluconolactone (the by product of oxidation of glucose by hexose oxidase).

In general halogenated furanones are the most popular compounds undergoing investigations of quorum sensing inhibition; their effect on bacterial biofilm has been observed in a wide range of Gram-negative bacteria (Smit 2004). The potential of quorum sensing inhibition is not only tested with antifouling perspective, but are also interesting for the medical industry, as substitution of other antibacterial agents (Smit 2004).

Table 1.10: Some of the most interesting furans from the study of Martinelli et al. (2004). The last three are the most inhibiting synthetic whereas the former are commercially available.

Sotolone	
Dihydroactinolide	
Methyltetrahydrofuranone	
Norfuranol	
L-ascorbic acid	
5-hydroxy-3-[(1R)-1-hydroxy-2,2-dimethylpropyl]-4-methylfuran-2(5H)-one	
5-hydroxy-3-(1-hydroxydecyl)-4-methyl-furan-2(5H)-one	
3-[(1R)-1-hydroxy-2,2-dimethylpropyl]-4-methylfuran-2(5H)-one	

Other natural antifoulants

The mechanism of the antifouling compounds extracted from nature differs greatly. Inhibition of sodium/potassium ATPase (Bufalin), anesthetic functionalities (pentyl 2-furyl ketone), repelants (tribromophenyl), is reported (Rittschof 2001), but also toxins, inhibitors of growth, and surface-energy modifiers are known. Some molecules can exhibit more than one biocidal effect. For example, inhibition of specific enzymes and of cellular messengers is an effect of vinocetine. Cyclic peroxides have amongst many other molecules been extracted from marine invertebrates (Placortis halichondroids) (Abarzua and Jakubowski 1995).

In general, the mechanisms exhibited by natural antifouling compounds, can be divided into toxic and non-toxic antifouling. For toxic antifouling the toxicity is limited by the bioavailability, and thus water solubility of the compounds. But also the relationship between rate of metamorphosis of the target organism, and the rate at which the compound kills, has a saying. For slow killing toxins, settlement and metamorphosis often precede death (Rittschof 2001). Non-toxic mechanisms include: disruption of attachment and metamorphosis, reversible anaesthesia and repulsion, and bacteriostatic activity. Complex molecules can often display several functionalities (Rittschof 2001). Though the mechanisms are called non-toxic, the increased time in the water column actually increases the probability of death in the fouling organisms (Rittschof 2001).
The mechanism of many extracted molecules, known to have antifouling effect, is not clear, often because of more than one possible interaction. This also applies the other way around; because one mechanism is known, other possible ways of action should not be ruled out (Rittschof 2001).

The major drawback, of the natural approach to environmentally friendly antifouling, is that the researchers are looking in rather exotic places and extracting from more and more exotic species. This means that if a successful compound is found, it could turn out to be very difficult to convert to commercial antifouling coatings. If a compound is found from an exotic species, it will be difficult to harvest. Chemical synthesis can turn out to be difficult and expensive. Therefore the route to commercialized antifouling coating can be prolonged when looking for natural antifoulers (Rittschof 2001). If/when a cheap way of producing the compound is discovered, it is also necessary to consider the potential toxicological effects. Just because the compound initially was found in nature, does not mean that it can be exploited excessively. New legislation on the area is very strict. Potential environmental consequences need to be taken into account, exactly as for synthetic compounds (Rittschof 2001) (see chapter two).

Known biocides

Some researchers are not focussing on the extraction of compounds from marine organisms. Food preservatives such as benzoate (Stupak et al. 2003) are amongst others a subject of investigation. Above water natural biocides such as tannins (Stupak et al. 2003) is also mentioned. Of the compounds listed in Table 1.7 quaternary ammonium salts (Baudrion et al. 2000) and isothiazolone (Willingham and Jacobsen 1996) has been tested for their potential antifouling activities.

The minimum inhibitory concentrations measured for the best performing quaternary ammonium salts was between 1 and 10 mg/L. Generally iodine is reported to be the best counterion in terms of inhibitory effect (Baudrion et al. 2000).

Willingham and Jacobsen report the antifouling ability of the isothiazolone, DCOI (4,5-dichloro-2-n-octyl-4-isothiazolin-3-one) to be very efficient. The LD_{50} is in the sub ppm level for barnacles, algae and diatoms. Because of a very short half-life in the marine environment (water and sediment) the compound is claimed to show a good environmental profile (Willingham and Jacobsen 1996).

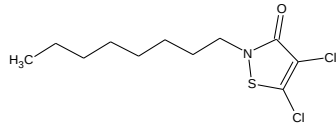


Figure 1.14: The structure of DCOI.

Preservatives

If one chooses to look to the food industry and the preservatives used there to find a potential antifouling agent, the benefits are easy to see. Food preservatives are well known thoroughly tested molecules. Under the slogan: “if you can feed it to humans, you can pour it into the oceans”, the route to political and commercial acceptance of an antifouling compound is (incorrectly) believed to be shortened significantly.

E-numbers are used in the European Union and Australia to mark commonly used food additives. Within the E-numbers there are approximately 30 different compounds that are used as preservatives (numbered in the interval E200-E299). It would be reasonable to start a search for environmentally benign antifouling compounds amongst these.

Table 1.11: List of E-numbered compounds used as preservatives. Adapted from: http://en.wikipedia.org/wiki/E_numbers#E200.E2.80.93E299_.28preservatives.29. Accessed February 2006..

E200 Sorbic acid	E233 Thiabendazole
E201 Sodium sorbate	E234 Nisin
E202 Potassium sorbate	E235 Natamycin, Pimaracin
E203 Calcium sorbate	E236 Formic acid
E210 Benzoic acid	E237 Sodium formate
E211 Sodium benzoate	E238 Calcium formate
E212 Potassium benzoate	E239 Hexamethylene tetramine
E213 Calcium benzoate	E240 Formaldehyde
E214 Ethyl para-hydroxybenzoate	E242 Dimethyl dicarbonate
E215 Sodium ethyl para-hydroxybenzoate	E249 Potassium nitrite
E216 Propyl para-hydroxybenzoate	E250 Sodium nitrite
E217 Sodium propyl para-hydroxybenzoate	E251 Sodium nitrate
E218 Methyl para-hydroxybenzoate	E252 Potassium nitrate
E219 Sodium methyl para-hydroxybenzoate	E260 Acetic acid
E220 Sulfur dioxide	E261 Potassium acetate
E221 Sodium sulfite	E262 Sodium acetates
E222 Sodium hydrogensulfite	E263 Calcium acetate
E223 Sodium metabisulfite	E264 Ammonium acetate
E224 Potassium metabisulfite	E270 Lactic acid (acid) (antioxidant)
E225 Potassium sulfite	E280 Propionic acid
E226 Calcium sulfite	E281 Sodium propionate
E227 Calcium hydrogen sulfite	E282 Calcium propionate
E228 Potassium hydrogen sulfite	E283 Potassium propionate
E230 Biphenyl, diphenyl	E284 Boric acid
E231 Orthophenyl phenol	E285 Sodium tetraborate (borax)
E232 Sodium orthophenyl phenol	E1105 Lysozyme

Of the preservatives listed in Table 1.11, the compounds related to benzoate (E210 -E219), Natamycin (E235) and Nisin (E234) are of interest. The former is tested in laboratory barnacle settlement tests and in field trials by Stupak et al. (Stupak et al. 2003). The tests described, is based on sodium benzoate, and they show that benzoate have an inhibitory effect of *Balanus amphitrite* (barnacles) nauplii for all the concentrations tested (25 - 75mM). Furthermore, the test showed antifouling effect on barnacles and "other benthic organisms" (Polydora ligni, Enteromopha and Ectocarpus) in sea trials of coating comprising sodium benzoate as sole biocide (Stupak et al. 2003). In another study by the authors, iron benzoate was tested for its effect on nauplii of a common barnacle (Balanus amphitrite). Stupak et al. concluded that the combined effect of benzoate and a marked pH decrease due to hydrolysis of iron benzoate produces a pronounced antifouling activity of the pigment (Perez et al. 2001). The effect of lowered pH and benzoate is found to be synergetic, which raise the question of the effect of benzoic acid compared to benzoate. A third study described by Stupak and co-workers (Vetere et al. 1999) present two possible mechanisms of the coatings

based on benzoate, the barnacle can either be repelled by the coating or become sluggish because of the narcotic effect. The same study reports lethal consequences of the pigment for a common fouling organism (Artemia salina). Finally no difference between the effect of calcium, aluminium and sodium benzoate is reported (Vetere et al. 1999).

Natamycin is also called picmarisin. It is a non-toxic antibiotic from streptomyces. It is a macrolide antifungal and has been used against yeast and mould. As a preservative it is very common in dairy products (Sarkar 2006).

Nisin is a polycyclic peptide comprising 34 aminoacid residues. It is produced by fermentation and is thus a natural preservative. It is highly specific and used both as a suppressor of Gram positive bacteria and as a selective agent in microbial media for isolation of gram negative bacteria, and yeast and moulds (Somogyi 2004). Not only Nisin, but also Nisin producing organisms are investigated in preservation of dairy products (Sarkar 2006).

Tannins are polyphenolic compounds with molecular weights in the range of 0,5 to 200kDa. They are very common in tea and wine. The toxicity towards micro-organisms can either involve enzyme inhibition, membrane interaction or metal ion deprivation. Tannins have been shown to induce morphology changes in several species of bacteria (Donovan and Brooker 2001). Stupak et al. has tested the antifouling effect of tannins extracted from chestnut, mimosa and quebracho. They all showed an inhibiting effect on settlement of Balanus Amphitrite nauplii in concentration ranges of ppm (Stupak et al. 2003). The antifouling efficiency in sea side tests is also reported. The coatings comprising tannins is reported to be effective in inhibiting settlement of primarilly barnacles over a period of four months (Stupak et al. 2003).

Hydrogen peroxide can be used in antifouling coatings, and the fate and effect of hydrogen peroxide is elaborated on in Appendix I. Table 1.12 summarises the effect of hydrogen peroxide on fouling organisms reported in the literature.

Furthermore, the list of potential environmentally friendly antifouling agents can be extended by the following hypothetically applicable: Isothiocyanates, plant phenolics, chitosan (and derivatives), trichorziner, adrenoceptor agonists, dextran, histatines.

Table 1.12: Summary of the reported effects of hydrogen peroxide on antifouling animals. The references are ordered chronologically.

Effective amount of H_2O_2	Test	Species	Reported effect	Reference
57.8- 578 mg/l	Chlorophyll reduction by bulk solution concentration.	Freshwater algae	Chlorophyll reduction to below 5 wt% after 24 hours.	Kay et al. 1982
1.36 mg/l, 1.7 mg/l	Cells exposed to the biocides in bulk solution for 24 h. Cell division rates monitored.	*Nitzchia closterium*, *Chlorella pyrenoidosa*. Marine algae	Cell division rate reduced to below 10 %. No effect on photosynthesis was identified.	Stauber and Florence 1987
0.7-1 ppm (35% H_2O_2 solution)	Presence of organisms on a net in a collector (test tube) with a water flow through. 90 days growth time.	Water taken from the sea. Abryozoa, Tunicates mussel polychaetes and malacostracans most abundant	Up to 90% settlement inhibition of the predominant species. Polychaetes and barnacle unaffected. Addition of iron(II) improve performance.	Nishimura et al. 1988
0.5-1.4 ppm (35% H_2O_2 solution)	Presence of organisms on a net in a collector (test tube) with a water flow through. 90 days growth time.	Water taken from the sea.	Reduction of organisms to 17.8 – 6.8 % of the control. Growth rate of mussels significantly inhibited at 2 ppm (0.35wt% solution). Fe(II) species improve the effect.	Ikuta et al. 1988
34 mg/l	Metamorphosis and settlement under laboratory conditions. Bulk solution H_2O_2 concentrations.	*Haliotis diversicolor*, abalone	Metamorphosis completely inhibited at 1 mM. Settlement approximately halved. Between 0.4 and 0.8 mM settlement and metamorphosis improved with increasing H_2O_2 concentrations.	Zhang et al. 2008
13.6 mg/l	Barnacle larvae settlement in bulk solution concentration assay. Experiment ended when 40% had settled.	Larvae from *Balanus amphitrite* (Barnacle)	0.4 mM hydrogen peroxide inhibits settlement. Catalase inverse the effect, pulsed electronic frequencies has better effect at lower hydrogen peroxide concentrations.	Perez et al. 2008

Test methods for antifouling agents.

Tests of novel biocides, with respect to application in antifouling coating systems can be divided into two groups; antifouling potency and environmental impact. With a practical antifouling point of view, the fouling inhibition tests are necessary, but with respect to legislation and politics, the environmental consequences of the compounds must be tested. Here, only the former group of test will be described, for the latter, there is referred to (Faimali et al. 2000).

The most common laboratory test of biocides for antifouling purposes is the barnacle (Balanus Amphitrite) test invented by Rittchof (1992) and described throughout the antifouling literature (e.g. (Stupak et al. 2003)). On site tests are typically performed using coatings containing the biocide in question (e.g. (Stupak et al. 2003)). The coatings are applied to panels mounted on

rafts beneath the surface of seawater. The former test is exclusively based on the concentrations of the biocide, and the latter requires detailed knowledge of the coating system and its release of biocide, knowledge seldom accessible for antifouling coatings under development.

Geraci and Faimali (2000) describe two different laboratory assays. The first is an assay based on Barnacles. A few tenths of larvae are placed in Petri dishes containing a seawater solution comprising the biocide. One Petri dish is needed for every concentration tested. The number of metamorphosed individuals is counted after a given amount of time (72 hours). The results are presented in terms of EC50 or LC50 numbers. The former is indicating the concentration at which the metamorphosis and settlement are inhibited in 50% of the individuals, and the latter is describing the lethal concentration for 50% of the individuals (Faimali et al. 2000). For biocides already included in a coating system, the tests are more plentiful. The paint can be applied on panels and exposed to seawater, or directly on ship hulls. It is more difficult in these cases to relate the antifouling effect, determined by inspection of the coated surfaces and estimation of percentages of surface that have been fouled, with the amount of biocide released. Seldom, the released amount of biocide is determined. If determined, one cannot be assured that the release rate has been constant throughout the exposure time.

Antifouling testing on blue mussels (a common fouling organism) can be achieved by the conventional barnacle settlement test or by adding the biocide to the foot of the mussel (Hellio et al. 2000). Hellio et al. (2000) extract phenol oxidase from the byssus gland of the blue mussel. The adhesive of mollusks is described in section 1 (Table 1.3). It involves the oxidation of phenol, catalyzed by phenol oxidase. Hellio et al. claim that inhibition of the enzyme will inhibit fouling by blue mussels effectively. Therefore they propose a screening method of potential antifouling compounds, which is based on inhibition of the phenol oxidase. The assay showed inhibition by the common antifouling agents, cuprous oxide and TBT. Some algal extracts were also tested positive for phenol oxidase inhibition (Hellio et al. 2000).

Antifouling efficiency of agents intended as quorum sensing inhibitors is more evolved. Mclean et al. (McLean et al. 2004) describe an assay where the compounds tested are included in an agar plate. Bacteria are placed on top of the agar. The bacteria used (Pseudomonas aureofaciens or Chromobacterium violaceum) produces pigments in a process regulated by quorum sensing. The mediator is N-hexanol homoserinelactone. Therefore absence of colour is indicating AHL inhibition by the compound tested (McLean et al. 2004).

Legislation

The following is meant as an introduction to the legislative mechanism, involving antifouling coatings in general, and the biocidal content more specificcally. First the European Union, Biocidal Products Directive will be covered, and afterwards will be given a brief description of the IMO.

Biocidal Products Directive (BPD)

The Biocidal Product Directive of the European Union (BPD-98/EC) is amongst 23 other product groups also governing the use of antifouling coatings. The definition of an antifouling coating, given in the directive, has been provided earlier in this text. The directive came into force May 14th 2000; the purpose of the directive is threefold (Pereira 2003):

1) to secure a high level of protection of man and the environment
2) to harmonize legislation concerning placing of biocidal products on the EU market
3) to remove barrier of trade within The European Union

The directive covers biocides as defined in the article 2(1a): "Active substances and preparations containing one or more active substances, put up in the form in which they are supplied to the user, intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means" (EU 1998). The terms: "render harmless, prevent the action of, or otherwise exert a controlling effect", is of special interest in relation to enzymatic antifouling. The consequence is that all products used for antifouling purposes should be treated equally. This is pointed out in the Directives Manual of Decisions in which, an enzyme known to act on the adhesive of biofouling organisms is concluded to be within the scope of the BPD (Manual of decision 1998).

Antifouling coating is one of 23 products covered by the directive. The coatings are defined as: "Products used to control the growth and settlement of fouling organisms (microbes and higher forms of plant or animal species) on vessels, aquaculture equipment or other structures used in water." (EU 1998). The BPD consists of 2 parts. The first stage is a review of existing active substances. During which active agents in antifouling coatings must be included into Annex 1. The second stage is authorization of the coatings containing the compounds treated in stage 1. Whereas the first stage is taking place within the framework of EU, the second stage is taking place at state level. After the implementation of the directive, only authorized biocides can be marketed. All biocidal compounds used as of 14/5 2000 are listed in Annex 1 in (EU 1998). The list comprises 953 substances (Pereira 2003).

Table 1.13: Compounds reported to be covered by data packs. I.e. Annex 3 of (EU 1998).

1)	Bis[1-cyclohexyl-1,2-di(hydroxyl-.kappa.O)diazeniu-mato(2-)]-copper
2)	Ziram
3)	Formaldehyde
4)	Sodium hydrogensulphite
5)	Disodium disulphite
6)	Sodium sulphite
7)	3-(4-isopropylphenyl)-1,1-dimethylurea/Isoproturon
8)	Diuron
9)	Thiabendazole
10)	Dodecylguanidine monohydrochloride
11)	Chlorotoluron
12)	Dimethyloctyldecyl[3-(trimethoxysilyl)propyl]ammonium chloride
13)	Cis-4-[3-(p-tert-butylphenyl)-2-methylpropyl]-2,6-dimethylmorpholine
14)	Fluometuron
15)	Lignin
16)	Copper thiocyanate
17)	Dicopper oxide
18)	Copper
19)	Poly-(hexamethylendiamine guanidinium chloride)
20)	Oligo-(2-(2-ethoxy)ethoxyethyl guanidinium chloride)
21)	Dichloro-N-[(dimethylamino)sulphonyl]fluoro-N-(p-tolyl)methane Sulphonamide/Tolylfluanid
22)	Captan
23)	N-(trichloromethylthio)phthalimide/Folpet
24)	Benzyl-C12-18-alkyldimethyl chloride
25)	Benzyl-C12-16-alkyldimethyl chloride
26)	Benzyl-C12-14-alkyldeimethyl chloride
27)	C12-14-alkyl[(ethylphenyl)methyl]dimethyl chloride
28)	Zineb
29)	Sulphur dioxide
30)	Potassium sulphite
31)	Dipotassium disulphite
32)	Chlorothalonil
33)	N'-tert-butyl-N-cyclopropyl-6-(methylthio)-1,3,5-triazine-2,4-diamine/Irgarol 1051
34)	Prometryn
35)	3-benzo(b)thien-2-yl-5,6-dihydro-1,4,2-oxathiazine,4-oxide
36)	4-bromo-2-(4-chlorophenyl)-1-(ethoxymethyl)-5-(trifluoromethyl)-1H-pyrrole-3-carbonitrile/Chlorfenapyr
37)	Iodine
38)	Pyrithione zinc
39)	Bis(1-hydroxy-1H-pyridine-2-thionato-O,S)copper
40)	Cetylpyridinium chloride
41)	Dichlofluanid
42)	Zinc sulphide
43)	Homopolymer of 2-tert-butylaminoethyl methacrylate
44)	Benzothiazol-2-ylthio)methyl thiocyanate
45)	4,5-dichloro-2-octyl-2H-isothiazol-3-one
46)	Chloromethyl n-octyl disulphide

Before May 1st 2006 data packs covering the environmental profile of the biocides intended for use in antifouling coatings must be handed to the European Union by the manufacturer of the biocides.

Antifouling compounds that have been reported to be covered by a data pack beforehand (annex 3 in (EU 1998)) are listed in Table 1.13. It is worth mentioning that neither enzymes, nor peroxides are among the substances listed.

Within 2 years of the deadline for submission of data packs, the European Union will provide a positive list of the substances authorized for antifouling use. The member states are thereafter responsible for the authorization of the products comprising the given compounds. The authorization of products can be done as frame formulations. For instance if coatings only differ in colour, they can be authorized in a frame formulation (Pereira 2003). Any compound that in the future should be utilized as active antifouling agent must be authorized first (i.e. listed in Annex 1). The expenditure for a manufacturer of biocides combined with addition of a new biocide to Annex 1 is estimated to be within 5-7 million Euros (Pereira 2003). These expenditures are primarily connected to the testing of the environmental impact, therefore known compounds that have undergone some degree of testing can be cheaper to verify. All new compounds must undergo such an evaluation, E-numbered preservatives, as well as brand new biocides or enzymes. As the directive is concerned with biocides having the active effect, it is still to be explored which parts of an indirect enzyme system with precursor system that must be validated as biocides.

$$\text{Substrate1} \xrightarrow{Enzyme1} \text{substrate2} \xrightarrow{Enzyme2} \text{biocide + biproduct} \qquad (1.9)$$

For the system depicted in equation (1.9), it is believed (by the legislation experts at Hempel A/S), that the enzyme 2 along with the biocide should be treated as a biocidal compound as described in the biocidal compound directive. A question to the "Manual of Decisions for Implementations of Directive 98/8/EC concerning the placing on the market of biocidal products" is being worked out. This should hopefully provide certainty on the subject.

The above described legislation process is aimed at a common legislation in the European community. The regulations are only concerning the application of the coatings, in the Member countries or on ships flying the flag of the member countries. It is therefore valid to produce, sell and export coatings that are not authorized. An exception of this is the TBT regulation [(EC) No 782/2003]. This regulation is purely concerned with TBT. Article 5.2 of the regulation states: "As from 1 January 2008 all ships flying an EU flag and all ships flying another flag that enter an EU port shall either not bear organotin compounds which act as biocides in anti-fouling systems on their hulls or external parts and surfaces, or bear a coating that forms a barrier to such compounds leaching from the underlying non-compliant antifouling system" (EU 2003). This means that from January 1st 2008 European ships must not actively use TBT based antifouling coatings (EU 2003).

This is an example of national legislation covering the waters of a given nation. Another example of national legislation is Alaska. Ships operating in Alaskan waters have not been allowed to be repainted with TBT containing antifouling-coatings according to an Alaskan state law of January 1st 2001. In Sweden copper-containing antifouling products are restricted on yachts according to geography. In the Baltic-sea area (eastern coast) all copper containing products are banned. On the western coast the leaching rate must be below a certain limit in the first month following immersion. The Netherlands has banned the use of copper-containing antifouling-coatings on yachts in fresh water areas (International 2003).

The International Maritime Organisation

The above discussion is only concerned with the national legislation in terms of the EU directive on biocides, and some exclusive national laws. Besides national regulations, the other main party playing an active part in the regulation of antifouling agents is the International Maritime Organisation (IMO). The IMO is a technical organization established in 1958 as an agency under the United Nations (IMO 2006). The governing body of IMO is the Assembly. The assembly meets every two years, in between meetings, the council, consisting of 40 Member states elected by the Assembly, acts as governing body (IMO 2005). Today there are 166 member states of IMO whose purposes are established in the first convention:

"To provide machinery for cooperation among Governments in the field of governmental regulation and practices relating to technical matters of all kinds affecting shipping engaged in international trade; to encourage and facilitate the general adoption of the highest practicable standards in matters concerning maritime safety, efficiency of navigation and prevention and control of marine pollution from ships". (IMO 2006)

IMO is empowered to deal with administrative and legal matters related to the purposes of the organization. The most important task for IMO is marine safety, but also pollution is a main subject. Around 40 protocols and convention has been adopted by the organization (IMO 2006). The most important of these conventions has been accepted of countries representing 98% of the world fleets. The Marine Environment Protection committee deals with pollution prevention (IMO 2005).

The International Convention on the Control of Harmful Antifouling System on Ships was adopted in 2001. It enters into force 1 year after 25 states representing 25% of the world's merchant shipping gross tonnage have ratified it (IMO 2001). Parties to the convention must make it national

law to prohibit and/or restrict the use of harmful antifouling systems on ships flying their flag, as well as ships operated under their authority and entering their ports, shipyards or offshore terminals. The content of the convention is to prohibit the use of harmful substances in antifouling coatings. The substances is listed in annex 1 of the convention which so far only involves organotin residues, but will be updated if/when necessary (IMO 2001).

Discussion

The preceding 57 pages are intended to deliver an overview of the process of fouling, its obstacles and the initiatives that are used to combat it. However, I am afraid to have failed in elucidating the truly complex and diversified nature of biofouling. It seems that only the most toxic of surfaces do not foul eventually after being immersed in the sea. However, innovative paint manufactures have thus far been able to delay the onset of biofouling on ship hulls of up to 5 years using increasingly more environmentally friendly antifouling coatings, and this tendency is continuing for the many years to come. Keeping in mind the alternative to efficient antifouling coatings (increased emission of green house gasses, loss of manoeuvrability, transport of marine organisms to new habitats, promoted corrosion of ship hull), it is evident that there is no alternative to combating biofouling. However, the means with which antifouling are combated should continuously become more environmentally friendly.

Replacing potent broad spectrum antifouling biocides with alternatives that are less harmful to non-target organisms becomes correspondingly more difficult because antifouling activity and toxicity often comes as a package. Environmentally benign antifouling can be obtained either by using very potent, but short lived biocides or biocides with a very narrow target area. However, considering the highly diverse environment that seawater is, and the pressure of fouling of tropical waters, it seems clear that several antifouling approaches should be combined in order to be able to substitute biocides of adverse long term effects to the marine environment.

References

Abarzua S, Jakubowski S. 1995. Review: Biotechnological investigation for the prevention of biofouling. I . Biological and biochemical principles for the prevention of biofouling. Marine ecology progress series. vol 123. p 301-312.

Allison D G. 2003. The biofilm matrix. Biofouling 19. p 139-150.

Ambio 2008.http://www.ambio.bham.ac.uk/about/what%20is%20biofouling.htm. Accessed October 2008.

Anderson C. 2000. Coatings, antifoulings. Kirtk-Othmer Encyclopedia of Chemical Technology. Vol 7. p150-167.

Anderson G. 2003. Seawater composition. Marine science. http://www.biosbcc.net/ocean/marinesci/02ocean/swcomposition.htm. Accessed february 2006

Anthoni U, Bock K, Christophersen C, Duus J Ø, Kjær E B, Nielsen P H. 1991. Structure of a novel antifouling epoxy cembrenoid diterpene from a sarchophyton. Tetrahedon 32. p 2825-2826.

Asghari A, Farra S R. 1993. Inactivation of bacteria by solids coated with magnesium peroxide. J. Environ. Sci. Health A28. p 779-793.

Baudrion F, Périchaud A, Vacelet E. 2000. Influence of concentration and structure of quaternary ammonium salts on their antifouling efficiency tested against a community of marine bacteria. Biofouling14. p 317-331.

Bers A V, Wahl M. 2004.The influence of natural surface microtopographies on fouling. Biofouling 20. p 43-51.

Bonaventura C, Bonaventura J, Hooper I R. 1991. Anti-fouling methods using enzyme coatings. Duke University. US Patent 5998200.

Brady R F, Drisko R W. 2000. Coatings, Marine. Kirtk-Othmer Encyclopedia of Chemical Technology 7. p192-206.

Chadwick I. 2005. The use of TAED/peroxide system. Speciality Chemicals magazine. p 30-31.

Clare A S, Rittschof D, Gerhart D J, Maki J S. 1992. Molecular approaches to nontoxic antifouling. Invertebrate reproduction and development. vol 22. p 67-76.

Clare A S, Rittschof D, Gerhart D J, Hooper I R, Bonaventura J. 1999. Antisettlement and narcotic action of analogues of diterpene marine natural product antifoulants from octocorals. Marine biotechnology 1. p 427-436.

Clark R J, Stapleton B L, Garson M J. 2000. New isocyano and isothiocyanato terpene metabolites from the tropical marine sponge acanthella cavernosa. Tetrahedon 56. p 3071-3076.

Class J B. 2000. Resins, natural. Kirtk-Othmer Encyclopedia of Chemical Technology. p 1-11.

Compere C, Bellon-Fontaine M N, Bertrand P Costa D, Marcus P Poleunis C, Pradier C M, Rondot B, Walls M G. 2001. Kinetics of conditioning layer formation on stainless steel immersed in seawater. Biofouling. vol 17. p 129-145.

Cooksey K E, Wigglesworth-Cooksey B. 1995. Adhesion of bacteria and diatoms to surfaces in the sea: a review. Aquatic microbial ecology. vol 9. p 87-96.

Costerton J W. 1999. Introduction to biofilm. Antimicrobial agents. 11. p 217-221.

Decho A W. 2000. Microbial biofilms in intertidal systems, an overview. Continental shelf research. vol 20. p 1257-1273.

de Kievit T R, Iglewski B H. 2000. Bacterial quorum sensing in pathogenic relationships. American society for microbiology. http://iai.asm.org/cgi/content/full/68/9/4839.

De la Court F H, De Vries H J. 1973. Leaching mechanism of cuprous oxide from antifouling paints. J oil colour chem assox. Vol 56. p 388-395.

Dempsey M J. 1981. Colonisation of antifoulng paints by marine bacteria. Botanica Marina 14. p 185-191.

Denyer S P. 1995. Mechanism of action of antibacterial biocides. International Biodeterioration & biodegredation. p 227-245.

Doose C A, Ranke J, Stock F, Bottin Weber U, Jastoff B. 2004. Structure-activity relationships of pyrithiones- IPC-81 toxicity tests with the antifouling biocide zinc pyrithione and structural analogs. Green chemistry 66. p 259-266.

Donovan L O, Brooker J D. 2001. Effect of hydrolysable and condensed tannins on growth, morphology and metabolism of *Streptococcus gallolyticus (S. Caprinus)* and *Streptococcus bovis.* Microbiology 147. P 1025-1033.

EC. 1998. Manual of decisions for implementation of directive 98/8/EC concerning the placing on the market of biocidal products. Available at: http://europa.eu.int/comm/environment/biocides/pdf/mod.pdf. Accessed February 2006

Egan B. 1987. Marine microbial adhesion and its consequences. In Sleigh M A ed. *Microbes in the sea.* John Willey & sons., Inc, New York.

Encyclopædia Britannica. 2008a. Barnacle. Encyclopædia Britannica Online. October 2008.

Encyclopædia Britannica. 2008b. Mussel. Encylcopædia Britannica Online. October 2008.

Encyclopædia Britannica. 2008c. Bryozoa. Encylcopædia Britannica Online. October 2008.

Encyclopædia Britannica. 2008d. Seaweed. Encyclopædia Britannica Online. October 2008.

EU. 1998. Directive 98/8/EC of the european parliament and of the council of 16 February 1998. Official Journal of the European Communities.

EU 2003.TBT regulation consequences. Directorate G - Maritime Transport and intermodality. Eurpean Commission Directorate - General for Energy and Transport.

Evans S M, Birhenough A C, Brancato M S. 2000. The TBT ban: out of the frying pan into the fire. Marine pollution bulletin 40. p 204-211.

Faimali M, Andrenacci M, Geraci S. 2000. Evaluation of antifouling biocide performance by means of laboratory test. European coatings 76. P 19-23.

Geske G D, Wezeman R J, Siegel A P Blackwell H E. 2005. Small molecule inhibitors of bacterial quorum sensing and biofilm formation. Journal of American chemical society. vol 127. p 12762-12763.

Grasshoff K. 1976. Methods of seawater analysis. Verlag Chemie.

Hawaii Biological survey. 2001. Available at: http://www2.bishopmuseum.org/HBS/invertguide/species_pdf/balanusamphitrite.pdf Accessed October 2008.

Hellio C, Bourgougnon N, le Gal Y. 2000. Phenoloxidase from the byssus gland of Mytilus edulis: Purification, partial characterization and application for screening products with potential antifouling activities. Biofouling16. p 235-244.

Hellio C, Thomas-Guyon H, Culioli G, Piovetti L, Bougougnon N, le Gal Y. 2001. Marine antifoulant from bifurcaria bifurcata (phaeophyceae, cysteoseiraceae) and other Brown macroalgae. Biofouling 17. p 189-201.

Ikuta S, Ichikawa S, Wakao Y, Nishimura K, Yasunaga T. 1988. Inorganic Peroxides for heat exchanger biofouling control. The American society of mechanical engineers.

IMO. 2006. http://www.imo.org/home.asp. Accessed February 2006.

IMO. 2005. Publications catalogue. 2005.

IMO. 2001. International convention on the control of harmful anti-fouling systems on ships, 2001. International Maritime Organisation.

International. 2003. Antifoulings the legislative position by country. Available at: http://www.antifoulingpaint.com/downloads/regulatory.pdf. Accessed February 2006.

Jackson S M, Jones E B G. 1988. Fouling film development on antifouling coatings with special reference to film thickness. International Biodeterioration 24. p 277-287.

Johannesen F. 2008. Kajak-strandvandring. Available at http://www.finn-j.dk/danmark/isefjord/ejby-apr04/index.html accessed october 2008.
Karlsson J, Eklund B. 2004. New biocide-free anti-fouling paints are toxic. Marine pollution bulletin 49. p 456-464.
Kiil S, Dam-Johansen K, Weinell C W, Pedersen M S. 2001. Analysis of self-polishing antifouling coatings using rotary experiments and mathematical modelling. Ind. Eng. Chem. Res. 40. p 3906 - 3920.
Kiil S, Dam-Johansen K, Weinell C E, Pedersen M S, Codolar S A. 2002a. Estimation of polishing and leaching behaviour of antifouling coatings using mathematical modelling: a litterature review. Biofouling 19. p 37 - 43.
Kiil S, Weinell C E, Pedersen M S, Dam-Johansen K. 2002b. Mathematical modelling of self-polishing antifouling coating exposed to seawater: a parameter study. IChemE 80. p 45 - 52.
Kiil S, Dam-Johansen, Weinell C E, Pedersen M S, Codolar S A. 2002c. Dynamic simulations of a self-polishing Antifouling coating exposed to seawater. Journal of coatings technology 74. p 54-54.
Konstantinou I K, Albanis T A, 2004. Worldwide occurence and effects of antifouling coating booster biocides in the aquatic environment: a review. Environment international 30. p 235-248.
König G M, Wright D. 1997. Laurencia rigida: Chemical investigations of its antifouling dichloromethane extract. journal of natural products 60. p 967-970.
Marinelli D, Grossmann G, Séquin U, Brandl H, Bachofen R. 2004. Effects of natural and chemically synthesized furanones on quorum sensing in chromobacterium. BMC Microbiology.
Marson F. 1969. Antifouling paints 1. Journal of applied chemistry 19. p 93-99.
Mathyasrasu J, Martiamutiu S, Muralidharan S, Meenakshisundaram R, Rengaswamy N S. 1997. Evaluation of peroxide based biocides for inhibition and biocidal efficiencies. Bulletin of Electrochemistry 13. p 289-293.
McLean R J C, Pierson III L S A, Fuqua C. 2004. Simple screening protocol for the identification of quorum signal antagonists. Journal of microbiological methods 58. p 351-360.
Melo L F, Pinheiro M M V P S, Bott R R, Pinheiro J D. 1988. Effect of design and operation variables. Fouling science and technology. p 223-232.
Melo L F, Bott T R. 1997. Biofouling in water systems. Experimental thermal and fluid science 14 p 375-381.
Miller M V, Bassler B L. 2001. Qurom sensing in bacteria. Annu. Rev. Microbiol. vol 55. p 165-199.
Monaghan C P, Kulkarni V H, Good M L. 1978. Release mechanisms of organotin toxicants from coating surfaces: A leaching model for antifouling coatings. Organometals and organometalloids. p 359-371.
Morris R S, Walsh I V. 1996. Zinc oxide photoactive antifoulant material. USpatent 5,916,947
Myers P. 2001. Polychaeta (on-line). Animal diversity web. Accessed October 2008 at http://animaldiversity.ummz.umich.edu/site/accounts/information/Polychaeta.html.
Novak J S, Fratamico P M. 2004. Evaluation of ascorbic acid as a quorum-sensing analogue to control growth, sporulation, and enterotoxin production in Clostridium perfringens. Food microbiology and safety 69. p 72-78.
Nishimura K. Yasunaga T, Ichikawa S, Wakao. 1988. Development of a new antifouling method for a marine cooling water system. Marine biology 99. p 145-150.
Okamura H, Aouama I, Takami T, Maruyama T, Suzuki Y, Matsumoto M, Katsuyama I, Hamada J, Beppu T, Tanaka O, Maguire R J, Liu D, Lau Y L, Pacepavicius G J. 2000. Phytotoxicity of the new antifouling compound irgarol 1051 and a major degradation product. Marine pollution bulletin 40. p 754-763

Okino T, Yoshimura E, Hirota H, Fusetani N. 1996. New antifouling kalihipyrans from the marine sponge acanthella cavernosa. journal of natural products 59. p 1081-1083.
Palagiano E, Zollo F, Minale L, Iorizzi M, Bryan P, McClinock J, Hopkins T. 1996. Isolation of 20 glycosides from the starfish Henrica downeyae, collected in the gulf of Mexico. Journal of natural products 59. p 348-354.
Pereria R C. Carvalho A G V, Gama B A P, Coutinho R. 2002. Field experimental evaluation of secondary metabolites from marine invertebrates as antifoulants. Braz. J. Biol 62. p 311-320.
Pereira M B. 2003. The EU biocidal products directive - a paint manufacturer's perspective. A/F conference, New Orleans. 2003.
Perez M, Garcia M, Vetere V, Deya M, del Amo B, Stupak M. 2001. Benzoates: a new approach to non-toxic marine fouling control. Pigment & resin technology 30. p 34-38.
Pettitt M E, Henry S L, Callow M E, Callow J A, Clare A S. 2004. Activity of commercial enzymes on settlement and adhesion of cypris larvae of the barnacle *Balanus amphitrite*, spores of the green alga ulva linza and the diatom navicula perminuta. Biofouling 20. p 299-311.
Redfield A C, Hutchins L W, Deevy jr E S. 1952. Marine antifouling and its prevention. United States Naval Institute.
Rittschof D, Clare A S, Gerhart D J, Mary S A, Bonaventura J. 1992. Barnacle in vitro assays for biologically active substances: toxicity and settlement inhibition assays using mass cultured Balanus amphitrite amphitrite Darwin. Biofouling, **6**(2). p 115-122.
Rittschof D. 2001. Natural product antifoulants and coating development. CRC press. 543-566.
Schultz M P, Swain G W. 2000. The influence of biofilms on skin friction drag. Biofouling 15. p 129-139.
Schultz M P. 2004. Frictional resistance of antifouling coating systems. Journal of fluids engineering 126. p 1039-1047.
Schultz M P. 2007. Effects of coating roughness and biofouling on ship resistance and powering. Biofouling 23:5. p 331-341.
Scott D A. 1990.Bronze disease: A review of some chemical problems and the role of relative humidity.Journal of the american institute for conservation 29. P 193-206.
Sera Y, Adachi K, Nishida F, Shizuri Y. 1999. A new sesquiterpene as an antifouling substance from a paulauan marine sponge, dysidea herbacea. Journal of natural products 62. p 395-396.
Smit A J. 2004. Medicinal and pharmaceutical uses of seaweed natural products: a review. Journal of applied phycology 16. p 245-262.
Somogyi L P. 2004. Food additives. Kirk-Othmer encyclopedia of chemical technology 12. P 29-34
Souza F D, Bhosle N B. 2003. Analysis of microfouling products formed on metallic surfaces exposed in a marine environment. Biofouling 19. p 95-107.
Somaseharan K N, Ubrammanian R V. 1980. Structure, mechanism and reactivity of organotin carboxylate polymers. Acs symp ser 121. p 165-181.
Stupak M E, Garcia M T, Pérez M C. 2003. Non-toxic alternative compounds for marine antifouling coatings. International Biodeterioration & biodegredation. Vol 52. p 49-52.
Tomono Y, Hirota H, Imahara Y, Fusetani N. 1999. Four new steroids from two octocorals. Journal of natural products 62. p 1538-1541.
Townsin R L. 2003. The ship hull fouling penalty. Biofouling 19. p 9-15.
Thomas K V, McHugh M, Hilton M, Waldock M. 2003. Increased persistence of antifouling coating biocides when associated with paint particles.. Environmental pollution 123. p 153-161.
van Wezel A P, van Vlaardingen P. 2004. Environmental risk limits for antifouling substances. Aquatic toxicity 66. p 427-444.
Vetere V, Perez M, Deya M, Stupak M del AMo B. 1999. A non-toxic antifouling compound for marine paints. Surface coatings international 123. p 586-589.

Sarkar S. 2006. Shelf-life extension of cultured milk products. Nutrition & Food Science 36. p 24-31.

Voulvoulis N, Scrimshaw M D, Lester J N. 1999. Review Alternative antifouling Biocides. Applied organometallic chemistry 13. p 135-143.

Voulvoulis N, Scrimshaw M D, Lester J N. 2000. Occurence of four biocides utilized in antifouling coatings, as alternatives to organotin compounds, in waters and sediments of a commercial estuary in the UK. Marine pollution bulletin 40. p 938-946.

Waite A J, Bonner J S, Autenrieth. 1999. Kintetics and stoichiometry of oxygen release from solid peroxides. Environmental engineering science 16. p 187-199.

Walker J T, Keevil C W. 1992. Nomarski DIC microscopy and image analysus of biofilms. Binary vol 4 p 92-95.

Weinell C E, Kiil S, Olsen K, Christoffersen M. 2003. Experimental study of drag resistance using a laboratory scale rotary set-up. International congress on marine corrosion and fouling 11. San Diego, Claifornia.

White D M, Irvine R L, Woolard. 1998. The use of solid peroxides to stimulate growth of aerobic microbes in tundra. Journal of hazardous materials 57. p 71-78.

Willingham G L, Jacobsen A H. 1996. Designing an environmentally safe marine antifoulant. In: DeVito S C, Garrett R L, Editors. *Designing safer chemicals: Green chemistry for pollution prevention.* ACS symposium series 640. p 225-233.

Wimpenny J, Manz W, Szewzyk U. 2000. Heterogenity in biofilms. Fems microbiology reviews 24. p 661-671.

Yebra D M, Kiil S, Dam-Johansen K. 2004. Review: Antifouling technology- Past, present and future steps towards efficient and environmentally friendly antifouling coatings. Progress in organic coatings 50. p 75-104.

Yebra D M, Kiil S, Dam-Johansen K, Weinell K. 2005. Reaction rate estimation of controlled-release antifouling paint binders: rosin based systems. Progress in organic coatings. Vol 53. p 256-275.

Yebra D M, Kiil S, Weinell C, Dam-Johansen K. 2006. American institute of chemical engineers. Vol 52 (5). p 1926-1940.

Yebra D M. 2005. Efficient and environmentally friendly antifouling coatings.Technical University of Denmark PhD thesis.

2. Chapter two – Enzymes in antifouling coatings

Enzymatic antifouling was first reported more than 20 years ago. Since then several patents describing the use of enzymes in antifouling coatings have been applied for. This chapter has been created in an effort to summarise the approaches done in order to achieve potent antifouling via the catalytic activity of enzymes. The chapter is divided according to the different effects intended by the applied enzymes, which will be defined in the introduction. Available information is limited by the commercial nature of the concept, which has resulted in patents being the primary source of information regarding enzymatic antifouling.

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Introduction

Enzymes are rapidly biodegraded proteins and they are therefore expected to be environmentally friendly. In fact, eco-toxicological investigations of three commercial enzyme products have concluded they were harmless to the environment (Allermann et al. 2004). It follows that if an efficient antifouling coating was developed based on enzyme technology as the primary antifouling mechanism, the environment would benefit from a decreased release of biocides to seawater.

The use of enzymes in antifouling coatings has been investigated continuously over the past 20 years. In this paper, the subject of enzymatic antifouling will be discussed in the light of the intended action of the enzymes applied, and the obstacles that need to be overcome to produce a successful product. It should be noted that patents are not peer-reviewed in the manner of scientific articles before publishing, and a patent does not indicate either its scientific value or technical content. However, almost all the knowledge available within this field has been published via patents or patent application and there is a general lack of knowledge regarding the theory behind enzymes as antifouling agents. The aim of this paper is to distinguish between established knowledge, intentions and hypotheses.

In the Biocidal Product Directive (BPD) of the European Union, antifouling coatings are defined as: "Products used to control the growth and settlement of biofouling organisms (microbes

and higher forms of plant or animal species) on vessels, aquaculture equipment or other structures used in water." (EU 1998).

Some antifouling coatings depend entirely on the physical properties of the surface for fouling control (i.e. fouling-release coatings) whilst the majority rely on the release of chemically active compounds i.e. biocides, to achieve inhibition of fouling (Yebra et al. 2004). Enzymatic antifoulings fall into the latter category, which is commonly divided into insoluble and soluble matrices, often referred to as self-polishing type coatings. The former releases its soluble pigments and biocides into seawater, but the binder constituents stay unaltered during operation, and the leached layer (the empty pores left by the dissolved pigments) therefore increases. Due to this increase in diffusion resistance, these coatings are known to have an inefficient utilisation of biocide, and a relatively low operational life-time (Redfield et al. 1952). Soluble matrices or self-polishing coatings are characterised by continuous release of binder constituents from the surface of the coating to the seawater along with the biocide release. This is referred to as polishing and results in a more stable thickness of the leached layer, which ensures a more constant biocide release rate and prolongs the lifetime of a coating (Yebra et al. 2004; Howell & Behrends 2006b).

The rate of polishing and biocide release of conventional coatings can be described by means of mathematical modelling (Kiil et al. 2001). The models provides a tool for coating engineers to assess the applicability of new coating materials (Kiil et al. 2002a), as well as the impact of seawater parameters (Kiil et al. 2002b, Yebra et al. 2006). Summarizing the studies, advanced antifouling coatings can control polishing and leaching rates by e.g. pigment dissolution, binder reaction with seawater, leaching of biocides, and friction forces from moving seawater. Therefore, if enzymes are applied in a highly complex, self-polishing coating, their impact on polishing behaviour can be damaging and enzyme-release thus uncontrolled.

From a commercial point of view, antifouling coatings must be available to the consumer, easily applied and prevent biofouling during the full service life of the coating (one year/season for yacht purposes). As a consequence of these requirements, any enzyme utilised must have at least the following qualities:

- Robust towards coating constituents.
- Non-destructive towards coating mechanisms.
- Broad spectrum antifouling effect.
- Stable activity in the coating and upon exposure of the coating to seawater

These requirements must be satisfied for an enzyme-based antifouling coating that is commercially successful.

Enzymatic antifouling

Enzymatic antifouling is used here to describe those antifouling coatings in which enzymes are an essential part of the biofouling inhibiting properties. Based on the intended action of the enzymes described in the patents and articles covered by this text, the mode of action for the antifouling effect can be divided into two groups, viz. direct and indirect antifouling. This distinction is based on the action of the enzymes.

Direct antifouling covers the use of enzymes, which actively interferes with fouling organisms. If the substrate of the enzyme is vital for the survival and well-being of the fouling organisms, the enzymes can be said to possess a biocidal effect, but if the substrate only affects the organism's ability to adhere to the surface, the enzyme is referred to as adhesive degrading.

Indirect antifouling covers the use of enzymes to provide the biocides actively inhibiting fouling. Their substrates can either be found in the surrounding environment i.e. seawater, or in-situ in the coating. Figure 2.1, the overall classification of the proposed mechanisms is depicted, and a schematic illustration of the mechanisms is shown.

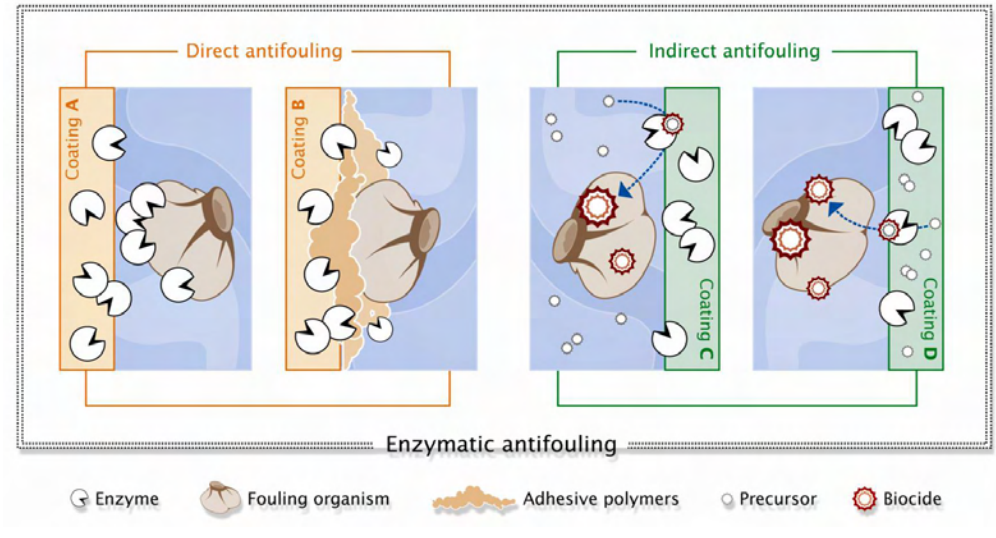

Figure 2.1: Proposed mechanisms of enzymatic antifouling. Coating A is based on biocidal direct antifouling. Coating B is based on adhesive degrading direct antifouling. Coating C is based on indirect antifouling with substrate in the environment. Coating D is based on indirect antifouling with the substrates provided from the paint.

Direct antifouling

Biocidal effect

Direct biocidal antifouling mimics the mechanism of action of commonly applied biocides. As illustrated in coating A in Figure 2.1, the mechanism is based on release of enzymes, which affect the viability of the fouling organisms. The direct biocidal mechanism of enzymes has only rarely been described. Table 2.1 shows the enzymes that have been proposed for direct biocidal antifouling.

Table 2.1: Data from the patents covering direct biocidal enzymatic antifouling. Percentages are by weight. DF = Dry film, WP = wet paint N.A. = not available.

Enzymes	Enzyme content	Binder	Test-methods	Pitfalls	Inventor
"cell wall degrading"	N.A.	Aqueous acrylic emulsion	Coated fishnets immersed in seawater for 4 months	Cationic and nonionic surfactants (20 wt%) and citric acid (0.1wt%) added as co-biocide	Kato (1987)
Chitinase Hyaluronidase Catalase	~0.1 wt% (WP)	Polyurethane	Panels exposed to seawater for 6 weeks.	Chitinase was primarily efficient towards barnacles. Hyaluronidase was not tested. Catalase was ineffective.	Bonaventura et al. (1991)
Chitinase Lysozyme	~5 wt% (DF)	Vinyl polymer, Rosin	Surface treated enzymes tested in coatings exposed to seawater.	Enzyme treatment increased polishing rate significantly.	Hamade et al. (1996)

Enzymes utilized in this as biocides include cell wall degrading enzymes (Kato 1987), lysozyme (Hamade et al. 1996), chitinase (Bonaventura et al. 1991) and hyaluronidase (Bonaventura et al. 1991) and all are expected to exert antifouling efficacy by mechanisms that are similar to more conventional biocides. Lysozyme is known to kill bacteria and is present in a number of human secretions, such as tears and saliva (Madigan and Martinko 2006). Chitinase catalyses the decomposition of chitin, which is an essential constituent of the barnacle exoskeleton (Bonaventura et al. 1991), and hyaluronidase catalyses the hydrolysis of hyaluronic acid that constitutes cell-tissue, increasing tissue permeability (Budavari et al. 1989). The effects of hyaluronidase (Bonaventura et al. 1991) and lyzosyme (Hamade et al. 1996) have not been established for fouling organisms, and the paper describing the effect of the cell wall hydrolyzing enzymes has not been translated into English (Kato 1987). The antifouling efficiency of chitinase against barnacles was pronounced, but several other biofouling organisms were not affected by chitinase (Bonaventura et al. 1991).

Catalase has also been applied in one occasion (Bonaventura et al. 1991). Catalase is known to catalyze the decomposition of hydrogen peroxide to water and oxygen (Madigan and Martinko

2006) and it is unsurprising that it did not contribute to antifouling activity of the coatings tested (Bonaventura et al. 1996).

Adhesive-degradation

Fouling organisms attach to a solid surface by various kinds of adhesives. The proposed effect of adhesive degrading enzymes is to hydrolyze these adhesives as shown in Figure 2.1 coating B. To successfully bond in seawater, an adhesive should be released rapidly from the settling stage of the organism i.e. cell, spore or larva, wet the surface thoroughly, be insoluble in seawater, and exclude water from the matrix during curing (Callow and Callow 2006).

Relatively little is known about the composition of the adhesives used by common fouling organisms, and new knowledge arises continuously. However, glycoproteins constitute the central part of the extracellular adhesives of spores of *Ulva*, a green algae (Callow and Callow 2006), carbohydrates are a major constituent of diatom adhesives (Chiovitti et al. 2006), and proteins are used by mussels (Sagert et al. 2006) and barnacles (Kamino 2006) as adhesives.

Antifouling accomplished by degradation of adhesives (Figure 2.1, coating A), may inhibit in more than one way. Besides repelling the organisms during the settlement stage, the effect of the enzymes may also be to release organisms after settlement by degradation of the adhesives used to anchor the cell/spore or larva to the substratum. However, since many of the adhesives used by fouling organisms 'cure' or cross-link, they would be expected to become less susceptible to enzyme degradation with time. Furthermore, for successful bonding to the substratum, the adhesive needs to exclude water in order to bond successfully (Callow & Callow, 2006). It is therefore expected that the primary effect of adhesive degrading enzymes, many of which are hydrolases and require water for activity, will be to either prevent settlement or weaken the attachment strength of the newly attached cells or organisms so they are swept away by hydrodynamic forces. It is unlikely that enzymes can be effective against adhesives that are already cured.

Adhesive degrading enzymes may interfere with the exploration stage of the settlement process. For example, barnacle cyprids larvae leave adhesive materials called 'footprints' of temporary adhesive as they explore the surface prior to settlement. This temporary adhesive differs from the cyprid cement or adult adhesive (see Bonaventura et al. 1991; Phang et al. 2006). If the enzymes applied degrade the temporary adhesive, their action may well contribute to the antifouling effect since the temporary adhesive also act as a settlement cue to exploring larvae (Clare et al. 1994). No footprints were seen when barnacle cypris larvae explored a surface in the presence of

serine-proteases and settlement was inhibited in a concentration-dependent manner (Pettitt et al. 2004).

Pettitt et al. (2004) provided the first conclusive evidence that the strength of adhesion of settled algal spores to the substratum could be decreased by the presence of hydrolytic enzymes. The earliest report of microbial control achieved by the action of enzymes was concerned with paper mills and water towers (e.g. Hatcher et al. 1970). In this application, the enzymes added to the cooling water were amylases. Since such dosing applications of enzymes are not relevant to their use in coatings they are not relevant to the present context.

Several patents concerning enzymatic adhesive degradation in antifouling coatings have been filed. Data gathered from the patents are presented in Table 2.2. The enzymes mentioned in the description of the invention are provided, as well as the binder system used. A column in Table 2.2 is dedicated to the pitfalls that have the potential to affect the information provided in the patent.

An enzyme-based antifouling coating was recently introduced by Allermann and Schneider (2006) to the Danish yacht market. According to an interview, the coating works by degradation of biofouling adhesives (Brejning 2006).

Broad spectrum effect

Based on the adhesives used by only four common fouling organisms (cf. above), it can be concluded that as a minimum, two types of enzymes must be applied for antifouling activity to be sufficiently broad, a protein-degrading and a polysaccharide-degrading enzyme. Several inventors have failed to consider the broad diversity of adhesives produced by fouling organisms and have only applied one type of enzyme (Noel 1984, Iwamura et al. 1989, Kuwamura et al. 1989, Okamoto et al. 1991). If a particular adhesive is not broken down by the enzymes present in the coating, the consequence may be heavy fouling by a few organisms, which may compromise the feasibility of direct adhesive-degrading antifouling.

Table 2.2: Data from the patents covering direct adhesive degrading enzymatic antifouling. The patents are listed chronologically. Percentages are by weight. DF = Dry film, WP = wet paint N.A. = not available.

Enzymes	Enzyme content	Binder	Test-methods	Pitfalls	Inventor
Protease B500 Protease A2 Maxazyme HP® Alcalase® Neutrase®	3-10 wt% (DF)	N.A.	Coated panels exposed to seawater for 9 months.	Citric acid and natamycin added as co-biocide 6-20 wt%	Noel (1984)
Cellulase Protease Cell-wall lytic	0.1-10wt% (DF)	Aqueous acrylic emulsion	Coated fishnets immersed in seawater for 4 months	Cationic and nonionic surfactants (20 wt%) and citric acid (0.1wt%) added as co-biocides	Kato (1987)
Protease 10P	20 wt% (DF)	Acrylate polymer	Coated nylon immersed in 12 months.	Abstract only in English	Iwamura et al. (1989)
Papain	11wt% (DF)	Polystyrene	Coated fishnets tested for antifouling ability	Abstract only in English	Kuwamura et al. (1989)
Protease	0.01-20wt%	Polyurethane	N. A.		Okamoto et al. (1991)
Serine protease Sulfhydryl protease Metallo protease Papaya protease Thermolysin Streptomyces protease Beta-amylase Beta-glucosidase Glycosidase Cellulase Pectinase Collagenase Beta-glucoronidase Trypsin Chymotrypsin Subtilisin Chymopapain Carboxpeptidase A Carboxpeptidase B	~0.1wt% (in examples)	Polyurethane	Panels exposed to seawater for 6 weeks.	Only a few of the proposed enzymes were tested: Chitinase, subtilisin, pronase, trypsin, α-glucosidase, β-glucosidase and catalase Short testing period.	Bonaventura et al. (1991)
Protease Hemicellulase Cellulase Lipase Amylolase	0.1 - 10wt% (0.5-2wt%)	Rosin/acrylic (solvent-based) Vinyl (Waterbased)	Coated panels exposed for 6 months in Elsinore (Denmark).	Geographical test location had low fouling intensity.	Allermann and Schneider (2000)
Protease	0.1-10 wt % (lipid coated enzyme)	Vinyl chloride Vinyl isopropyl ether WW rosin.	Coated panels immersed in seawater for 12 months.	Enzyme treatment increased polishing rate significantly.	Hamade et al. (1996)
Glucose oxidase Proteases alpha-amylase Lipase	N.A.	Polysiloxane Polyurethane acrylate	Laboratory assays on enzyme-solutions and -coatings.	Fouling intensity in laboratory is different from that of the sea.	Huijs et al. (2004)

Enzyme mobility

The degree of enzymatic mobility i.e. potential to be moved freely is relevant with regards to maintaining efficient adhesive degradation. Enzymatic mobility is often caused by leaching of the enzyme(s), which creates an enzyme solution in the boundary water layer outside the coating (Allermann et al. 2004; Bonaventura et al. 1991). Insufficient antifouling activity was the result of too fast enzyme leaching in one case (Allermann et al. 2004).

Enzymes attached to the binder material of the coating (i.e. immobilized in the coating) have been made and effective inhibition of settlement was reported (Huijs et al. 2004). This indicates that only limited mobility of the enzymes is needed for the coatings to work in accordance with a direct adhesive degrading mechanism.

Enzyme distribution

If the leaching-rate of enzymes is very low (i.e. enzymes are effectively immobilized), the distribution of enzymes in the coating should be addressed (Bonaventura et al. 1991). A distribution of 1000 Å between adjacent enzymes is stated to be sufficient for good antifouling performance, but 100 Å is preferred. There are no technical investigations supporting these figures, but if the 100 Å postulate is considered, the weight of active enzyme corresponds to 0.035 wt% (with a dry film density of 1.5 kg/litre). This means that no theoretical limit is set due to the content of enzyme in a coating. Their stability, however, may be limiting.

Polyurethane was the binder used in one of the patents recorded in Table 2.2 and it is difficult to see how enzyme-release from thermoset polyurethane coatings can occur. Unless the coating is formulated above the critical pigment volume concentration (where the coating becomes porous and thus compromises other coating attributes such as mechanical properties), the antifouling effect reported is brought about in effect by immobilized enzymes (Bonaventura et al. 1991).

Biocidal enzyme additives

Commercial enzyme-additives can contribute to the antifouling effect of a coating. When enzymes are purchased, they typically contain a large amount of stabilizing additives and/or preservatives. The amount of enzyme is therefore only a small fraction of the material, and the additives may also possess biofouling inhibititory properties.

Pettitt et al. (2004) reported an equal effect of heat denatured enzymes and native enzyme solutions for some commercial enzyme products. This effect was shown for settlement of spores of *Ulva linza* in solutions of Alcalase 2.5L type DX® (a protease), and settlement and mortality of barnacle cyprids (*Balanus amphitrite*) in solutions of AMG 300L® (an amylase). The content of Alcalase 2.5L Type DX® was 5.7 % protein (Pettitt et al. 2004), and the additives consisted, amongst other substances, of sorbitol and calcium formate (Allermann et al. 2004). AMG 300L® contains 4.4 % protein and additives are potassium sorbate, sodium benzoate and sucrose/glucose (Allermann et al. 2004). Though both enzyme products are established not to be harmful to the environment (Allermann et al. 2004), sodium benzoate has specifically been tested for antifouling efficacy on *Balanus amphitrite* (Stupak et al. 2003). In the case of Alcalase®, formate may have an inhibitory effect as formic acid is reported toxic to mice and human skin (Budavari et al. 1989).

Alcalase® 2.5L and AMG 300L® are used widely in the literature (Allermann and Schneider 2000, Schasfoort et al. 2004, Pettitt et al. 2004). The results reported for studies using formulations of commercial enzyme should therefore be interpreted with caution.

Environmental impact of direct enzymatic antifouling

If the proposed mechanism of adhesive degrading enzymatic antifouling holds true, the environmental consequences of incorporating enzymes into marine coatings should be minimal. The motility of algal spores and the mortality of barnacle cyprids are reported to be unaffected by certain commercial enzyme products (Pettitt et al. 2004). Three commercial enzyme products have specifically been tested for their impact on the marine environment (Allermann et al. 2004) and the results were positive in all three cases. From the ecological studies performed (Allermann et al. 2004), it seems valid to assume that the major environmental impact of commercial enzyme products would be due to compounds added as stabilisers and/or preservatives.

Antifouling effect of direct adhesive degrading antifouling

In the first patent covering the composition of an antifouling coating that contained enzymes (Noel 1984), the data indicated better performance of the novel enzyme-based coatings compared to that of two commercially available antifouling coatings. The test period of nine months in natural seawater indicated long-term potential of the invention, but the mixture of enzymes and biocides make it difficult to interpret the results clearly with respect to the effect of the enzymes.

Another enzyme-based coating (Kato 1987) claimed efficient control of biofouling when applied to fishnets. However, the addition of surfactants and carboxylic acid to improve performance makes it difficult to determine the effect of the enzymes. Though the enzymatic effect is not established, it is interesting that slime was controlled by the formulation reported.

The patented binder described by Iwamura et al. (1989) claims higher antifouling efficiency than a commercial binder when enzymes are added to both. Thus, the effect of the enzymes is masked by the overall antifouling effect of the coating. A patent by the same authors (Kuwamura et al. 1989) described physical immobilization of protease onto a polystyrene polymer. According to the English abstract, good antifouling efficiency was achieved when it was applied to nylon. Another patent (Okamoto et al. 1991) has been issued based on the antifouling activities of proteases, but the English abstract provides little information about the antifouling effect, so it will not be discussed further here.

Until the early 1990s, the types of enzymes incorporated into antifouling coatings were limited to proteases, amylases, and cellulases. The patent by Bonaventura et al. (1991) listed a range of enzymes for their potential use in antifouling coatings. Besides proteases, enzymes that degrade chitin, various polysaccharides, biological tissue and glycoproteins were included. The majority of these were intended to contribute to the degradation of the extracellular matrix of the organisms making up the biofouling community, but only a few of them were tested for antifouling activity. Based on a study in which the different coatings were exposed to seawater for 42 days, the authors concluded that proteases and chitinase inhibited biofouling significantly. However, the effect was reported to be species-specific.

The actual antifouling effect of enzymes has been proven in laboratory assays, where the enzyme-product is added to the bulk-solution. Allermann and Schneider (2000) used knowledge from such laboratory assays to select the enzymes applied in trials of antifouling coatings. Although the effect of the enzymes had been shown it is likely that the coatings themselves exhibited considerable antifouling efficacy.

Enzymatic stability and compatibility in solvent and coating matrices was the aim of the patent by Hamade et al. (1996). Surface treatment of hydrolytic enzymes with glucoxide derivatives was proven to significantly increase lipase activity in toluene and coatings. The lipid treatment however, increased the polishing rate. Since the influence of the polishing rate on the antifouling effect was not evaluated, it is possible that the antifouling performance reported was due to faster polishing. Again, proof regarding the actual effect of the enzymes was lacking.

Covalent attachment of enzymes to functional groups of a polymer in the coating showed antifouling activity in laboratory assays of coatings employing diatoms and barnacles (Huijs et al. 2004). Generally speaking, the enzymes contributed significantly to the antifouling effect of the coating. It was shown that immobilized enzymes were superior to dispersed enzymes in terms of antifouling potency.

Indirect enzymatic antifouling

Substrates from surroundings

Enzymes in an immersed antifouling coating can act by converting compounds in seawater into potent antifoulants (Figure 2.1, coating C). Two patents have described the conversion of naturally occurring compounds into antifouling agents by enzymes enclosed in the antifouling coating. The scope of these patents is presented in Table 2.3.

Table 2.3: Data from patents covering indirect enzymatic antifouling where the substrate is present in the environment. Percentages are by weight. DF = Dry film, WP = wet paint N.A. = not available.

Enzymes	Reaction catalysed	Enzyme content	Binder	Test-methods	Pitfalls	Inventor
Haloperoxidase	$H_2O_2 + Br^- \rightarrow HOBr + OH^-$	~0.01 wt% (WP)	Acrylic latex Chlorinated rubber Polyacrylamide	Solutions with and without haloperoxidase were tested for antifouling efficacy. HOBr release from coatings was proven.	Content of hydrogen peroxide in natural seawater is lower than the amount used in trials.	Wever et al. (1994)
Malate oxidase Glucoseoxidase Hexoseoxidase Cholesteroloxidase Arylalcoholoxidase Galactoseoxidase Alcoholoxidase Lathosteroloxidase Aspartateoxidase Amnio acidoxidase Amineoxidase D-glutamateoxidase Ethanolaminoxidase NADHoxidase Urate-oxidase Superoxid dismutase 1 - 10 % (DF)	Substrate + O_2 → Product + H_2O_2	1 - 10 wt% (DF)	Polyvinyl acetate Acrylic Polyurethane Rosin Alkyd	Antifouling test of enzyme-coatings on rafts.	Proteases as precursor enzyme will degrade the other enzymes in the coating. Incomplete conversion of precursor product may counteract the antifouling effect.	Schneider and Allerman n 2002

Hypohalogenic acid (e.g. HOBr) is an oxidizing acid of halogens, which is produced from halide ions and hydrogen peroxide in a reaction catalyzed by haloperoxidase. The seawater content of hydrogen peroxide and halide ions can be converted into hypohalogenic acid by haloperoxidase present in an antifouling coating (Wever et al. 1994). Hypohalogenic acid is a potent antifoulant; the effect of the acid exceeding that of a corresponding amount of hydrogen peroxide (Wever et al. 1994). However, a major drawback of the invention is the availability of hydrogen peroxide in natural seawater. In antifouling tests performed, hydrogen peroxide was added in amounts of at least 10 µM (Wever et al. 1994), but the hydrogen peroxide content of surface seawater rarely exceeds 0.1 µM (Yuan and Shiller (2001).

Hydrogen peroxide-producing oxidases were proven to have efficient antifouling activity during "monthly" inspections (Schneider and Allermann 2002). The substrates for the oxidases were not present in the surroundings, but via a precursor enzyme, suspended proteins (proteases) or polysaccharides (amylases) in the seawater were converted into substrates for the oxidase. However, the inventors failed to account for the risk of adding proteases to another enzyme system since proteases would be expected to degrade the other enzyme present in the coating.

Substrates from within the coating

Enzymes can be applied as a way of controlling the release of biocides. In this review, such a mechanism is referred to as indirect antifouling where the substrates are part of the coating (Figure 2.1, coating D). Data from patents and applications based on this approach to enzymatic antifouling are shown in Table 2.4.

In contrast to the direct approach, indirect enzymatic antifouling varies more in the type of enzymes and reactants. The substrate/enzyme/product can be chosen from several different enzyme technologies. The fact that a substrate must be added to the coating makes the water solubility of the substrate a parameter of concern since if the substrate is very water-soluble, it will leach out of the coating rapidly (Kiil et al. 2002b).

A variety of enzymatic reactions producing possible antifouling agents have been proposed (Hamade et al. 1997). One or all of the products can be proposed as antifouling agents (c.f.Table 2.4). A coating composition comprising tricaprin (C_{10} fatty acid trilipid) and lipase is the only example of indirect antifouling where the substrate comes from the coating, and for which data are provided (Hamade et al. 1997). A coating exposed to seawater for three months was reportedly free of "slimy biofouling". A similar coating, containing capric acid instead of the enzyme system, was

described as fully fouled. The difference in efficacy may be explained by the controlled release of capric acid from the former coating, and fast leaching of capric acid from the latter, but it may also indicate that the enzyme system itself had antifouling activity. Although the data are inconclusive, the two tests indicate that the antifouling effect is due to the enzyme system and not because of the binder or surface properties (Hamade et al. 1997), a consistency seldom seen in the examples of the patent literature.

Table 2.4: Data from the patents covering indirect enzymatic antifouling where the substrate is provided from the coating. *n is the original number of glucose residues in starch.

Enzymes	Reaction catalysed	Products	Binder	Pitfalls	Inventor
Esterase Amidase Alcoholdehydrogenase Chitosanase	$RCOOR' + H_2O \rightarrow RCOOH + R'OH$ $RCONHR' + H_2O \rightarrow RCOOH + R'NH_2$ $RCOH + O_2 \rightarrow RCO + H_2O_2$ decompostion of chitosan	Acid Alcohol Amine Peroxide Aldehyde Chitosan-decomposition-products	Acrylate	Lipase-system tested for antifouling properties. Capric acid assumed to be the active ingredient.	Hamade et al. (1997)
Hexose oxidase Amyloglycosidase	$C_6H_{12}O_6 + O_2 \rightarrow C_6H_{10}O_6 + H_2O_2$ $starch_n + H_2O \rightarrow C_6H_{12}O_6 + starch_{n-1}$*	Hydrogen peroxide Gluconolactone	Commercial A/F coating without biocide	H_2O_2 release from coatings established. No antifouling data. Incomplete conversion of precursor product may counteract the antifouling effect.	Poulsen and Kragh (1999)

The composition of an enzyme-based antifouling coating is described in more detail by Poulsen and Kragh (1999). The focus of this patent was to provide a continuous release of hydrogen peroxide from the coating. The release mechanism was divided into an initial one-step process followed by a two-step process. The former comprises the hydrogen peroxide releasing system, while the latter includes a precursor enzyme-system producing the substrate for the first step. The coating leached hydrogen peroxide in a stable manner (Poulsen and Kragh 1999) and the invention is claimed to be effective at preventing fouling. However, data substantiating this claim were not provided.

Substrate instability

Substrates incorporated into a coating must be present in a sufficient amount for the enzymes to continuously deliver an effective dose of the antifouling product. The concentration should preferably not fluctuate on a geographical scale or be time dependent.

Substrates added to the coating are initially required in a sufficiently high enough amount for the coating to have an efficient release rate during the operational time of the coating. High amounts may be incompatible with other paint constituents or with paint production in general. A continuous release of the substrate is needed for the continued control of fouling and is usually achieved by precursor enzyme-systems. In these cases (e.g. starch to glucose) a complete turnover of the precursor product is necessary, as it is expected that the release of some enzyme substrates (e.g. glucose) can counteract the release of antifouling agents.

Enzyme activity

Manipulation of enzyme activity may be necessary when dealing with indirect antifouling where the coating contains one of the substrates. In these cases it is important to ensure that no conversion of the substrates occurs before the coating is immersed. This may be done by eliminating one or more of the substrates needed in the conversion (e.g. O_2, H_2O) in the paint can. In the absence of co-substrates, the content of the paint will be stable. Once immersed in seawater, penetration of these substrates into the coating will then induce substrate conversion.

Other aims of enzymatic activity in coatings

Enzyme-containing coatings may be used for purposes other than antifouling activity. In one case, they have been added to facilitate polishing of the coating (Schasfoort et al. 2004). In another scenario, phenol oxidizing enzymes were used to cure lignin (Bolle and Aehle 1993), which imparted antifungal properties to the resulting coating (Felby and Hansen 1996). In a more exotic application, enzymes incorporated into coatings were used to decompose a series of organophosphorus compounds (McDaniel 2006).

Microbial antifouling

Living micro-organisms and hydrolytic enzymes have been applied in antifouling coatings on at least two occasions (Selvig et al. 1996, Polsenski and Leavitt 2002).
Table 2.5 presents selected information from these patents. The addition of micro-organisms is supposed to provide several benefits. Firstly, the added micro-organisms will compete with biofouling micro-organisms for nutrients. Secondly, "benign" micro-organisms can excrete antibiotics or active compounds that hinder colonisation of the surface (Dobretsov et al. 2006). Thirdly, the coating would be expected to contain hydrolytic enzymes produced by the micro-organisms (Selvig et al. 1996). Microbial antifouling has also been used in combination with enzymes in the coating (Polsenski and Leavitt 2002).

Table 2.5: Content of patents based on microbial antifouling.

Micro-organism	Enzymes	Binder	Test	Conclusions	Applicant
Bacillus sp. *Escherichia sp.* *Pseudomonas sp.*		Liquid polymer Polyurethane Silicone Acrylic latex	4 months immersion of coated panels.	Antifouling performance increased with time.	Selvig et al. (1996)
Bacillus sp. *Escherichia sp.* *Pseudomonas sp.* Yeast Fungi	Protease Amylase Cellulase Lyase Hydrolase	Acrylic	Hydrolysis by coatings.	Hydrolytic activity increased with increasing number of coating layers and addition of nutrients in sub-layers.	Polsenski and Leavitt (2002)

Spores and vegetative forms of micro-organisms have been evaluated for antifouling efficacy (Selvig et al. 1996). When the coatings are compared to an uncoated control, it appears that the presence of micro-organisms delays the onset of fouling. The micro-organisms used are usually rod-shaped gram-positive bacteria, the delayed antifouling effect being explained by their dormant endospores (Madigan and Martinko 2006). When coatings containing micro-organisms are applied in layers, hydrolytic activity is increased with an increase in the number of coating layers (Polsenski and Leavitt 2002). The addition of nutrients to the micro-organisms in the sub-layers of the coating is also beneficial for the hydrolytic activity of the coating (Polsenski and Leavitt 2002).

Discussion

Legislative aspects of antifouling agents, and hereunder enzymes were considered in chapter one. The chronological development of patents described in the preceding paragraphs is presented in Figure 2.2. The literature was found by searching the databases; Scifinder, Web of Science and

Espacenet. As a time indicator, the priority date was chosen, but it should be remembered that patent-applications are published with 18 months delay.

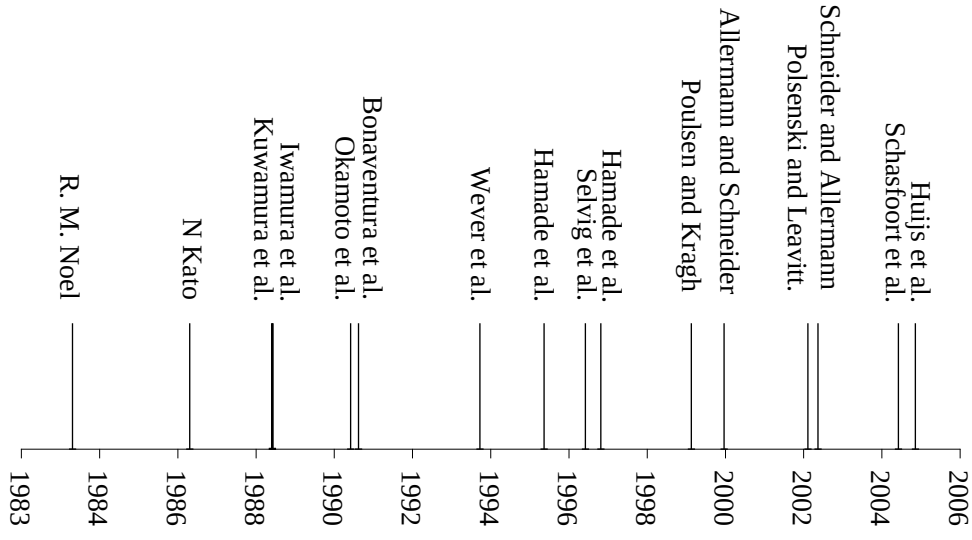

Figure 2.2: Timeline on the historical development of patents describing enzymatic antifouling.

Figure 2.2 shows that patenting is spread almost evenly over the years since the technology was first invented in the early 1980s, indicating continuing research on enzyme-based antifouling compositions. Given the date and the results of the first patent describing enzymatic antifouling (cf. above), it may come as a surprise that no antifouling product was commercialized earlier.

Based on the patents described above, the major obstacles connected to the development of an enzyme-based antifouling coating, which can fulfil the expectations of the consumers are presented in Table 2.6 and will be discussed further below.

The pitfalls and unknowns are the major focus. It should be kept in mind that the benefits of a successfully developed coating would have little or no environmental impact on non-target marine organisms, no possibility of long term bioaccumulation, and a safer working environment for the work-force involved with paint application.

Table 2.6: Overview of the obstacles connected to development of enzymatic antifouling coatings.

General obstacles for enzyme-based antifouling	

• Knowledge of the enzyme products impact on fouling species
  o Alternate effects of the enzymes
  o Biocidal effect of enzyme additives
  o Overlapping of direct and indirect antifouling
• Practical issues
  o Combining enzymes and paint ingredients
  o Legislative issues
  o Impact of enzymes on coating properties
  o Stability of enzymes	

Obstacles connected to the direct approach	Obstacles connected to the indirect approach

• Necessary enzymatic distribution
• Necessary enzymatic mobility
• How to achieve broad spectrum efficiency
• Enzyme stability on surface
	• How to keep the enzymes inactive during storage
	• How to activate the enzymes upon immersion
	• Amount of biocide that can be generated
	• Dependency of substrates
	  o Necessary amount/concentration
	  o Stabilization of substrate supply
	  o Complete substrate turnover

Evaluation of effect

When enzyme-based antifouling coatings are evaluated for their antifouling performance, not all effects may be known. The enzyme itself may act on more than one substrate whilst additives in the enzyme product may possess antifouling properties. Furthermore, coating parameters, such as polishing rate and surface properties, which may be affected by the addition of enzymes, also have a significant effect on the antifouling properties. The effect of paint colour on short term antifouling activity was also recently established to be significant (Swain et al. 2006). A substantial time frame and the inclusion of appropriate controls should therefore be used when assaying the antifouling ability of enzyme-based coatings in the marine environment.

Supplementing effects of the enzymes

The enzymes involved in the inhibition of biofouling may degrade substances other than those intended. The settling stages of several organisms explore the surface prior to settling leaving footprints behind, which may differ in composition from the permanent adhesive(s) secreted once settlement has occurred (Bonaventura et al. 1991). The number of barnacle cyprid footprints is significantly reduced by active proteases (Pettitt et al. 2004), but how much impact this would have in terms of subsequent colonisation remains to be determined.

Enzymatic activity and stability

Decomposition of enzymes is inevitable and generally follows a first order reaction mechanism (Goodfrey & West 1996). Adding enzymes into the paint could therefore prove inexpedient. Optimization of enzymatic stability in the paint solvent and matrix has been addressed (Hamade et al. 1996), while degradation by bacteria may also negate the benefits of using proteinaceous compounds. In addition, as recognized by Poulsen and Kragh (1999), enzymes have relatively narrow niches of activity within pH, temperature and salinity. Considerations regarding stability of enzyme activity must therefore be addressed when developing enzyme-based antifouling coatings.

A mix of enzymatic antifouling agents is often applied to address the diversity of fouling organisms, and many of the enzyme blends applied contain proteases (Schneider and Allermann 2002, Allermann and Schneider 2000). Since enzymes are proteins, proteases will self-degrade. The stability and potential self-degradation of enzymes should be kept in mind when estimating the concentration of enzymes needed for a required activity over time.

Overlapping effects

Distinction between indirect and direct enzymatic antifouling may prove to be impossible. Two very similar enzymes have been used for indirect antifouling (hexose oxidase (Poulsen & Kragh 1999)) and direct antifouling (glucose oxidase (Huijs et al. 2004)). In the latter case, the enzymes were immobilized but still efficient antifoulants. Thus, it cannot be ruled out that the antifouling effect claimed in the former patent is due to a direct antifouling effect of hexose oxidase.

For practical purposes, the mechanism of action is inconsequential but for environmental assessment, the mode of action is critical. If the activity of an enzyme is restricted to the extracellular adhesives of the biofouling-organisms, the environmental impact is limited. On the other hand, if the activity is due to toxicity, the environmental impact is also dependent on the enzymes' lifetime in seawater.

Enzymes impact on coating behaviour

Enzymes are relatively new coating constituents. In cases where substrates must be provided in the coating (i.e. indirect antifouling), a large fraction of the coating may be a novel ingredient. The activity of enzymes will also depend on the solvents/diluents of the paint and for solvent-based systems; enzymatic denaturation may be accelerated by unfolding of the enzyme.

The desired lifetime of an antifouling coating will often exceed the lifetime of an active enzyme situated at the surface of the coating. The enzymes at the surface should therefore be continuously replenished by fresh enzyme during operation. Replenishment of active enzyme will most likely be achieved through a self-polishing coating. For a coating to obtain or retain its polishing behaviour it is necessary to evaluate the novel "pigments" (substrates or enzyme containing particles). Kiil et al. (2002a) provided a tool to determine the compatibility of a new soluble pigment in a self-polishing antifouling coating (i.e. to produce suitable polishing and leaching rates when adding a novel pigment). Using physical data (seawater solubility and density) of the novel pigment and an approximate diffusion coefficient of the dissolved pigment in seawater, an estimate of the polishing and leaching rates for a self-polishing antifouling coating can be obtained from dimensionless figures (i.e. without solving the mathematical model provided in Kiil et al (2002a)). The technique can also be used to estimate the seawater solubility of a novel soluble pigment that one should aim for to obtain a certain polishing and leaching behaviour (Kiil et al. 2002b).

Conclusions

A list of what is documented within the field of enzymatic antifouling is tabulated below:

- Proteolytic enzymes reduce settlement of barnacle cyprids, reduce the number of cyprid footprints and reduce the adhesion strength of algae
- Hydrolytic enzymes are active antifoulants in bulk solution
- Hydrolytic enzymes are active antifoulants in coatings
- Enzymes immobilized in the coating are reported to have antifouling activity
- Enzymes can be applied to facilitate controlled release of other compounds
- An enzyme-based antifouling coating is available on the Danish yacht market

The main challenge of enzyme-based antifouling technology is how to retain activity for a realistic i.e. commercially viable, period of time. In several cases, concepts as well as short term antifouling activity in coatings have been proven, but long term efficiency towards all fouling organisms remains to be reported. This obstacle can be ascribed to difficulties related to paint production, storage, and operation. Based on the limited number of commercial products available, simply mixing enzymes and paint is ostensibly insufficient, and therefore some sort of enzymatic stabilization and controlled release must be achieved for the concept to fulfil its potential.

Enzymes applied to inhibit fouling within the European Union, will be considered as biocides and will therefore have to undergo registration similar to that for conventional biocides.

References

Allermann K, Schneider I, Walstrøm E, Andersen B H, Andersen T T, Højenvang J. 2004. Substitution af biocider i bundmaling til skibe med enzymer. Danish Environmental protection Agency. Miljøprojekt Nr 910 [in danish] Available at: http://www.mst.dk/ [INTERNET].

Allermann K, Schneider I. 2000. Antifouling paint composition comprising rosin and enzyme. US patent Application Publication. US2002/0166237A1.

Allermann K, Schneider I. 2006. Coatzyme. Product of Biolocus A/S. Available at: http://www.biolocus.com/. Accessed January 2007.

Bickerstaff G F. 1997. Immobilization of enzymes and cells. Humana Press Inc. ISBN13: 978-0-89603-368-3

Bolle R, Aehle W. 1993. Lignin based paint. United States Patent number:6072015

Bonaventura C, Bonaventura J Hooper I R.1991. Anti-fouling methods using enzyme coatings. United States patent number 5998200.

Brejning J. 2006. Ny dansk bundmaling med enzymer (in Danish). Available at: http://www.baadmagasinet.dk/online/content/v3/index.php?SectionID=1&ContentID=5355&1140714479. Accesed February 2007.

Budavari S, O'Neil M J, Smith A, Heckelman P E. 1989. The Merck Index eleventh edition. New York: Merck & Co. Inc.

Callow J A., Callow, M E. 2006. The *Ulva* Spore Adhesive System. In: Smith A M, Callow, J A, editors. Biological Adhesives. Springer Verlag Berlin Heidelberg p 61-78.

Chiovitti A, Dugdale T M, Wetherbee R. 2006. Diatom Adhesives: Molecular and Mechanical Properties. In: Smith A M, Callow, J A, editors. Biological Adhesives. Springer Verlag Berlin Heidelberg p 79-103.

Clare A S, Freet R K, Mclary M J. 1994. On the antennular secretion of *Balanus amphitrite amphitrite* and its role as a settlement pheromone. J Mar Bio Assoc UK 74: 243-250.

Dobretsov S, Dahms H-U, Qian P-Y (2006) Inhibition of biofouling by marine microorganisms and their metabolites. Biofouling 22: 43-54

Felby C, Hansen T T. 1996. Process for impregnating solid wood and product obtainable by the process. European patent specification EP0934142B1

Goodfrey T, West S. 1996. Industrial enzymology 2nd ed. Stockton press/Macmillian press ltd. ISBN 0-333-59464-9

Hamade K R, Yamammori T N, Yoshio K O. 1996. Glucoxide derivatives for enzyme modification, lipid-coated enzymes, method of producing such enzymes and antifouling paint composition. US patent 5770188.

Hamade R, Kadoma-dhi, Yamammori, Kyotanabe-shi. 1997. Method for controlled release of compounds having antimicrobial activity, and coating composition. US patent 6150146.

Hatcher H J, Truda R J,Lechner T G, Elmo L, McDuff C R. 1970. Sätt och komposition för att motverka slembilding i vatten. Swedish patent SE375347.

Howell D, Behrends B. 2006a. A review of surface roughness in antifouling coatings illustrating the importance of cutoff length. Biofouling 22: 401-410

Howell D, Behrends B. 2006b. A methodology for evaluating biocide release rate, surface 22: roughness and leach layer formation in a TBT-free, self-polishing antifouling coating. Biofouling p 303-315.

Huijs F M, Klijnstra J W, van Zanten J. 2004. Antifouling coating comprising a polymer with functional groups bonded to an enzyme. European patent application EP1661955A1.

Iwamura G, Kuwamura S, Shoji A, Kito K. 1989. Antifouling coating materials containing immobilized enzymes. Japanese patent JP02227471.

Kamino K. 2006. Barnacle Underwater Attachement. In: Smith A M, Callow, J A, editors. Biological Adhesives. Springer Verlag Berlin Heidelberg. p 145-166.
Kato N. 1987. Enzyme-containing antifouling emulsion coating compositions. Japanese patent JP63202677.
Kiil S, Dam-Johansen K, Weinell C E, Pedersen M S. 2001. Analysis of self-polishing antifouling paints using rotary experiments and mathematical modelling. Ind Eng Chem Res. 40. P 3906-3920.
Kiil S, Dam-Johansen K, Weinell C E, Pedersen M S, Codolar A C. 2002a. Dynamic simulations of a self-polishing antifouling paint exposed to seawater. Journal of coatings technology 74. P 45-54.
Kiil S, Dam-Johansen K, Weinell C E, Pedersen M S. 2002b. Seawater-soluble pigments and their potential use in self-polishing antifouling paints: simulation based screening tool. Progress in organic coatings 45. P 423-434.
Kuwamura S, Iwamura G, Shoji A, Kito K. 1989. Antifouling coatings containing enzymes. Japanese patent JP02227465.
Madigan M T, Martinko J M. 2006. Cell structure/function. In: Madigan M T, Martinko J M, editors. Brock Biology of Micro-organisms 11th ed. Person Prentice Hall. P 55-101.
McDaniel C S, McDaniel J, Wales M E, Wild J R. 2006. Enzyme-based additives for paints and coatings. Progress in organic coatings 55. p 182-188.
Noel R. 1984. Composite anti-salissure pour adjonction aux revêtements des corps immergés et revêtemenet la contenant. French patentFR2562554.
Oamoto K, Shiraki Y, Yasuyoshi M. 1991. Coating compound composition. Japanese patent JP19910026643
Pettitt M E, Henry S L, Callow M E, Callow J A, Clare A S. 2004. Activity of commercial enzymes on settlement and adhesion of cypris larvae of the barnacle balanus amphitrite, spores of the green alga ulva linza nd the diatom navicula perminuta. Biofouling 20. p 299-311.
Phang Y, Aldred N, Clare A S, Callow J A, Vansco G J (2006) An in situ study of the nanomechanical properties of barnacle (*Balanus amphitrite*) cyprid cement using atomic force microscopy (AFM). Biofouling 22: 245-250.
Polsenski M J, Leavitt R I. 2002. Coatings with enhanced microbial performance.US patent application US2004/0009159.
Poulsen C H, Kragh K M. 1999. HOx as antifouling. US patent application US2002/0166237.
Redfield A C, Hutchins L W, Deevy jr E S.1952. Marine antifouling and its prevention. United States Naval Institute.
Sagert J, Sun C, Waite J H. 2006. Chemical Subtleties of Mussel and Polychaete Holdfasts. In: Smith A M, Callow, J A, editors. Biological Adhesives. Springer Verlag Berlin Heidelberg p 125-144.
Schasfoort A, Eversdijk J, Allermann K, Schneider I. 2004. Self-polishing anti-fouling coating compositions comprising an enzyme. PCT application WO2006/002630A1.
Schneider I, Allermannn K. 2002. Antifouling composition comprising an enzyme in the absence of its substrate. US patent application US2005/0147579A1.
Selvig T A, Leavitt R I, Powers W P. 1996. Marine antifouling methods and compositions. US patent 5919689.
Stupak M E, Garcia M T, Pérez C. 2003. Non-toxic alternative compounds for marine antifouling paints. International Biodeteterioration & Biodegradation 52. p 49-52.
Swain G, Herpe S, Ralston E, Tribou M. 2006. Short-term testing of antifouling surfaces: the importance of colour. Biofouling 22. p425-429.

Wever R, Dekker L H, Van Schijndel J W P M, Vollenbroek E G M. 1994. Antifouling paint containing haloperoxidases and method to determine halide concentrations. PCT application WO95/27009.

Yebra D M, Kiil S, Dam-Johansen K. 2004. Review: Antifouling technology - Past, present and future steps towards efficient and environmentally friendly antifouling coatings. Progress in organic coatings 50. p 75-104

Yebra D M, Kiil S, Weinell C E, Dam-Johansen K. 2006. Mathematical modeling of tin-free chemically-active antifouling paint behavior. American Institute of Chemical Engineers Journal 52. P1926-1940.

Yuan, A M Shiller. 2001. The distribution of hydrogen peroxide in the southern and central Atlantic ocean. Deep-Sea Research II 48. pp 2947 - 2970.

3. Chapter three - Inorganic precursor peroxides for antifouling coatings

In this chapter utilisation of inorganic peroxides as precursors for hydrogen peroxide is described. The aim has been to identify the applicability of inorganic peroxides as antifouling coating ingredients, and determine the antifouling efficiency of coatings containing the compounds. First will be given a short introduction, where previously reported uses of inorganic peroxides are described, and the mechanism of polishing will be refreshed; then the materials and methods applied will be described, and finally the results will be provided followed by a discussion of the perspectives of the use of inorganic peroxides as active antifouling coating ingredients.

The M.Sc studen Ajish John has contributed significantly to the data provided in this chapter. Sincere thanks for hard work and thorough reporting. The content of this chapter has been published in the Journal of Coating Technology and Research with the title ‘Inorganic precursor peroxides for antifouling coatings.’ (Authors: Olsen S M, Pedersen L T, Hermann M H, Kiil S, Dam-Johansen K).

Introduction

In the search for more environmentally friendly solutions to the biofouling challenge, antifouling coating development is ongoing in industry and academia. A potentially very attractive alternative to traditional biocide-based antifouling is to utilise hydrogen peroxide as active ingredient. Such a product would not suffer from environmental drawbacks due to the rapid decomposition of hydrogen peroxide in seawater into water and oxygen as shown in equation (3.1):

$$2H_2O_2\,(aq) \rightarrow 2H_2O(l) + O_2\,(aq) \qquad (3.1)$$

Antifouling based on inorganic peroxides has previously been described in two Japanese patents dating back to 1988 (Minuro et al. 1988) and 1989 (Minuro et al. 1989). In both cases, the applicant is Chugoku Marine Paints ltd. According to the abstracts, which are the only part of the papers available in English, zinc peroxide and strontium peroxide are applied as pigments, and carboxylated acrylic copolymers are used as binder material. Antifouling performance comparable to cuprous oxide is claimed. However, to our knowledge, no product based on this technology has been commercialised, and because access to data in the patents is very limited (patent in Japanese), we believe a systematic investigation into the application of inorganic peroxides as active

ingredients in polishing antifouling coatings are of value to the community working on developing tomorrows antifouling solutions.

The intention of the work described in this paper is to establish the potential of peroxides of magnesium, calcium, strontium and zinc as active ingredients in polishing antifouling coatings.The results have shown that if substituting the commonly applied seawater-soluble pigment, zinc oxide for zinc-peroxide, antifouling performance of a fast polishing coating is improved.

Polishing of antifouling coatings enables maximal effect of the biocides applied in the coating. Compounds leaching from an insoluble coating leave behind a leached layer growing in thickness over time. According to Fick's first law of diffusion, the reduced gradient caused by the increased diffusion distance decreases the flux of biocide over the interface between binder and seawater, and consequently the impact of the biocide on the fouling organisms (Yebra et al. 2004). The result is shorter operation time for the coating. By continuously polishing off the outermost binder of the antifouling coating, the effect of a given amount of biocide can be optimised, and operational time of the coating is increased (Yebra et al. 2004). Polishing is achieved by a continuous release of the outermost binder material into seawater during operation of the ship. This requires that the binder is partly soluble in seawater (i.e. seawater soluble binder- based polishing), or that it can react with seawater to become soluble (chemically reacting binder) (Yebra et al. 2004).

Polishing is a mechanism depending on the physical and chemical properties of the binder matrix, as well as the pigments (Kiil et al. 2002). Detailed insight into the mechanisms involved in polishing, and the important parameters affecting the rate of polishing have been provided by Kiil et al. (2002) and Kiil et al. (2001) (see chapter one). Pigment parameters influencing polishing rates include, shape, particle size distribution, diffusion coefficients of dissolved species, and rate of dissolution. Generally antifouling coatings for yachts must stay efficient for one year. However, for commercial ships, the coating is expected to last for 3 to 5 years.

Hydrogen peroxide cannot be applied directly as ingredient in an antifouling coating. It is only accessible in aqueous mixtures, highly reactive and hazardous in combination with organic material and it will leach out of antifouling coatings too rapidly. If hydrogen peroxide is to become the active part of an antifouling coating, it is necessary to generate it in-situ. Hydrogen peroxide can be generated by enzymatic reactions (Olsen et al. 2007), photo-catalytic reactions (Morris and Walsh 1996), or it can be a product of a reaction between water and a suitable precursor compound such as inorganic peroxides (Steiner and Eul 2001).

The only reports of hydrogen peroxides antifouling effect are concerned with laboratory assays based on biofouling organisms exposed to hydrogen peroxide concentrations as a bulk solution. Settlement of mussels is inhibited in 0.7 ppm hydrogen peroxide (Ikuta et al 1988), and the effect is enhanced by the presence of iron(II) ions (Nishimura et al. 1988).This effect is caused by the iron ions catalytic effect on decomposition of hydrogen peroxide to hydroxyl radicals, these radicals are more powerful oxidants than hydrogen peroxide (Elzanowska et al. 1995). For a more elaborate discussion on hydrogen peroxide the reader is referred to Appendix I.

The reaction between inorganic peroxides and water yields hydrogen peroxide or molecular oxygen, or a combination thereof (Waite et al. 1999). Solid peroxides are used as oxygen providers in bioremediation (Waite et al. 1999) or generally as stimulants of growth of aerobic microbes (White et al. 1998). Antibacterial effects are documented for several types of peroxo compounds, such as sodium perborate (Mathyarasu et al. 1997), sodium peroxide (Mathyarasu et al. 1997), and magnesium peroxide (Asghari and Farrah 1993). Peroxides may be formed from all groups in the periodic system, and in addition to peroxides, peroxyhydrates, peroxopolyoxometallates, superoxides, and ozonides are known peroxo compounds (Steiner and Eul 2001). Provided in Table 3.1 are some of the peroxo compounds commonly used in industrial processes and products. Due to relatively slow reaction with seawater, limited solubility, suitable peroxide content, and commercial accessibility, the peroxides of magnesium, calcium and strontium and zinc was preferred for testing as antifouling coating ingredients.

Another type of peroxo compounds that can be used to generate hydrogen peroxide in situ in antifouling coatings is organic peroxides. However, no organic peroxides have been included in the experimental part of this paper. This is due to high water solubility of these organic compounds. The suitable dimensionless seawater solubility, α, should have a value between 10^{-7} and 10^{-9} for a pigment to be used in self-polishing antifouling coatings (Kiil et al. 2002). Dibenzoyl peroxide dimensionless water solubility is $6.8*10^{-6}$ and for peracetic acid, α is $8.8*10^{-6}$ (water solubility and not seawater solubility has been applied in both cases). Furthermore, organic peroxides have several safety issues, which limit their use due to exposure to humans during coating production and application.

When using precursors to provide active antifoulants, it is uncertain whether the precursor or the compound generated should be treated as the active compound in terms of legislation. However, as hydrogen peroxide is the compound intended to actively hinder fouling, we believe that hydrogen peroxide is the prime “biocide” and should therefore be registered if inorganic peroxides are

intended for use in antifouling coatings. To our knowledge, hydrogen peroxide has not been registered as active antifouling agent in the Biocidal Product Directive of the European Union (BPD-98/EC).

Table 3.1: Selected inorganic peroxides, their water solubility, safety issues and use in modern industry. N.A.: data not available.

Compound/formula	Safety issues	Uses
Strontium peroxide SrO_2	-Powerful irritant to skin and mucous membrane -Strong oxidizer, causes fire on contact with combustible substances	-Pyrotechnics - Bleaching agent
Sodium perborate tetrahydrate $NaBO_3 \cdot 4H_2O$	-Oxidant, undergoes hazardous decomposition	-Detergents and cleaning product- ingredient - Bleaching agent
Sodium percarbonate $(Na_2CO_3)_2 \cdot H_2O_2$	-Skin irritant, oxidizer	Cleaning product ingredient
Sodium perborate $NaBO_3$	-More stability compared to other peroxides due to the presence of peroxygen bonds	-Antiseptic/disinfectant -Detergent ingredient -Eye drop ingredient
Calcium peroxide CaO_2	Causes irritation to mucous membrane, eyes and skin on exposure	-Rice treatment -Water treatment
Magnesium peroxide MgO_2	Causes irritation to mucous membrane, skin and eyes.	-Bleaching -Disinfecting -Deodorizing -Bioremediation of contaminated soil
Zinc peroxide ZnO_2	Powerful irritant to skin, eyes and mucous membrane	-Bleaching -Curing

Strategy of investigation

The aim of this work has been to determine the potential of inorganic peroxides as active antifouling coating ingredients. Based on theoretical evaluations, the peroxides of magnesium, calcium, strontium, and zinc have been selected for testing. To characterise the compounds as potential pigments, particle size distribution, and critical pigment volume concentration (CPVC) values have been obtained.

It has been a priority to establish the potential of the peroxide as the pigmentation in a polishing antifouling coating. Therefore polishing has been monitored for all the peroxides in three different coating compositions. Fast-, intermediate- , and slow- polishing binder composition was chosen as model coatings. Antifouling efficiency has been established based on a laboratory assay including barnacle larvae, and panels immersed in seawater.

Experimental Methods and Materials

Calcium-, and magnesium -peroxide were obtained from Solvay as Ixper 75C ®, and Ixper 35M ® respectively, and strontium- and zinc -peroxides were purchased from Sigma Aldrich. In Table 3.2, the available data on the commercial peroxides have been summarised.

Table 3.2: Properties and measured characteristics of the four peroxides selected for practical testing.

	Magnesium peroxide	Calcium peroxide	Strontium peroxide	Zinc peroxide
Formula	MgO_2	CaO_2	SrO_2	ZnO_2
Cas nr	1335-36-8	1305-79-9	1314-18-7	1314-22-3
Peroxide content (wt%)	37±2	78±2	100	50-60
Residual (supplier information)	MgO and $MgCO_3$	Other inorganic calcium compounds.	None	ZnO
Density of product (g/ml)	3.00*	2.90*	4.56	2.9 *
M of peroxide content (g/mol)	56.3	72.1	119.6	97.4
Oil absorption (g oil/100 g pigment)	24±1	38±3	32±2	31±2
Critical PVC (%)	56±1	45±2	39±1	53±2
n(H_2O_2)/V(MO_2) (mol/ml)	0.019	0.031	0.038	0.016
n(H_2O_2)/V(AF coating) (mol/ml) For PVC=CPVC	0.011	0.014	0.015	0.009

*Values from Steiner and Eul (2001)

Coating formulation

Water would induce reaction of the inorganic pigments in the can, and therefore only solvent-based coatings were considered for the inorganic peroxides, and limited resources and space on the test facilities limited the number of coatings included in the test series.

Three different model antifouling coating-types were produced. The coatings represented slow, intermediate and fast polishing coatings respectively, and they varied primarily in the ratio between the binder constituents. The binders were composed of a water soluble part, a retarder, and a plasticiser. In all the coatings, the water soluble content came from zinc resinate (produced from rosin (Cas No: 65997-06-0) and commercial grade zinc oxide), and the retarder was acrylate (methyl methacrylate/n-butyl methacrylate/methacrylic acid terpolymer purchased as Degalan LP 64/12, from Rohm GMBH). The plasticiser in the fast and intermediate polishing coatings was polyether (Lutonal M40, 45% from BASF AG), and in the slow polishing system, diisodecyl phthalate (CAS: 68515-49-1) was used as plasticiser. The aim was to produce coatings of the same λ (λ=PVC/CPVC). However, for the intermediate polishing binders, coatings with a high level of λ

were also produced. Table 3.3 is a complete list of the coatings produced. As references, similar binder systems containing cuprous oxide and/or zinc oxide were produced. However, for the reference coatings, only λ=0.6 was used. Coatings were produced on two-speed Diaf 37-33v mixer at low speed, using a dissolver turbine disk of 5 cm in diameter, and 250 ml cans with a diameter of 6.5 cm. Dispersing was complete when the liquid paint reached fineness of grind below 60 µm.

Table 3.3: List of experimental coatings. The numbers refer to the binder or amount of pigmentation. The binder constituents represent fast, slow and intermediate polishing antifouling coatings.

Classification		Pigmentation			Binder composition (Solid volume of binder part of the film)		
Name	Expected polishing rate	Inorganic peroxide	PVC	λ	Seawater soluble part	Plasticiser	Retarder
#1Ca	Fast	CaO_2	29.0	0.65	82.5	17.5	
#1Zn	Fast	ZnO_2	29.0	0.58	82.5	17.5	
#2Mg	Intermediate	MgO_2	33.6	0.60	82	14	4
#2Ca	Intermediate	CaO_2	27.0	0.60	82	14	4
#2Sr	Intermediate	SrO_2	22.5	0.60	82	14	4
#2Zn	Intermediate	ZnO_2	30.0	0.60	82	14	4
#3Ca	Slow	CaO_2	27.0	0.60	58	7	35
#3Sr	Slow	SrO_2	22.5	0.60	58	7	35
#3Zn	Slow	ZnO_2	30.0	0.60	58	7	35
#4Ca	Intermediate	CaO_2	43.0	0.96	82	14	4
#4Zn	Intermediate	ZnO_2	50.0	0.94	82	14	4
#1reference	Fast	ZnO	21	0.61	82.5	17.5	
#2reference	Intermediate	70 vol% Cu_2O 30 vol% ZnO	43	0.64	82	14	4
#3reference	Slow	80 vol% Cu_2O 20 vol% ZnO	40	0.60	58	7	35

The experimental pigments were tested for coating compatibility, and formulated coatings were used to determine the performance of the inorganic peroxides in antifouling coatings in general.

The following is a list of the experiments performed to establish the coating compatibility and antifouling performance of the inorganic peroxides.

Critical pigment volume concentration

The critical pigment volume concentration (CPVC) was estimated from the peroxides oil absorption values (Wicks 2002). The oil absorption was measured in triplicate by adding commercial grade linseed oil to a known amount of particulate until a paste was obtained. The oil absorption was calculated as the amount of oil needed to wet 100 g of particulate, and CPVC was established using equation (3.2)

$$CPVC = \frac{1}{\frac{OA \cdot \rho(pig)}{100 \cdot \rho(oil)} + 1} \quad (3.2)$$

Particle size distribution

The particle size distributions of the inorganic peroxide were measured based on the formulated liquid paints. A Mastersizer 2000 from Malvern Instruments, and a Hydro 2000 G sample disperser were used. A few drops of the liquid paint was taken and dispersed in commercial grade xylene, and particle size distribution was measured directly on the slurry.

Water immersion test

The effect of water on the cured coatings was monitored in a laboratory test. Coatings were applied on 5 x 10 cm polycarbonate panels and the panels were immersed in tap water adjusted to 45 °C. Low salinity and increased temperature were used to accelerate potential failures of the coatings. The panels were inspected visually and monitored gravimetrically, once a week during five weeks. Before weighing, excess water was gently wiped off the panels.

Hydrogen peroxide release rates

The release rates of hydrogen peroxide from the inorganic peroxide-containing coatings were measured by establishing the accumulation of hydrogen peroxide in a constant volume of water added to Petri dishes coated with the experimental coatings. Glass Petri dishes (diameter of 6 cm) were primed in order to ensure adhesion between experimental coating and glass. Two-component Hempadur 4518 from Hempel A/S was used as primer, because it adheres well to smooth surfaces. The experimental peroxide-containing coatings were applied one day after the primer, and as application tool, a brush was used. The coatings were conditioned in artificial seawater for one day before initiation of tests.

Hydrogen peroxide concentrations were measured using a Merckoquant® peroxide test from Merck (1.10011.0001) with a detection range from 0.5 to 25 mg/l. The initial establishment of hydrogen peroxide release rates were performed after one day, and several measurements were done spanning three hours. After five and ten days further conditioning in artificial seawater, the

hydrogen peroxide concentration after 15 minutes of accumulation in freshly applied artificial seawater was established.

In addition to the colorimetric determination, hydrogen peroxide concentrations were determined spectrophotometrically using a coupled enzyme assay based on 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). ABTS reacts with hydrogen peroxide in a peroxidase-catalysed reaction, and hydrogen peroxide concentrations can therefore be indirectly determined by measuring the light absorption at 405 nm in an assay adapted from Savary et al. (Savary et al. 2001). 50 µl was sampled from the Petri dishes for each concentration measurement and a calibration curve ranging from 0.001 to 0.05 mM was used to convert absorption to hydrogen peroxide concentration.

Before measuring the release rates, the Petri dishes were removed from the container with artificial seawater (ASW), and washed. The accumulation of hydrogen peroxide was measured in unused water (ASW or deionised). The time allowed for hydrogen peroxide to accumulate in the water ranged from minutes to hours. Before sampling the Petri dishes were gently stirred. The UV-measurements in artificial seawater were performed after 20 days conditioning in artificial seawater, and those in deionised water were done after 21 days conditioning.

Polishing and leaching

Polishing and leaching characteristics were measured using a rotary set-up similar to the one described by Kiil et al. (2001). The pH was adjusted frequently to 8.2 using 1 M sodium hydroxide or 1 M hydrochloric acid. The rotor was operated at 20 knots during the experiment.

Samples were prepared by coating overhead transparencies that had been primed using Hempadur 4518 from Hempel A/S to improve adherence to the smooth transparency film. Coating samples were applied adjacent to each other using a multiple Dr Blade applicator with a gap of 250 µm. However, due to viscosity differences, individual application of #4Ca, # 2Sr and #2reference was neccesary. For these a gap size of 350 µm was used. After curing, the coated transparency was cut in strips of 2 cm resulting in samples of 1.5 x 2 cm. The strips were mounted on the rotor, and after having been immersed for the intended time, the samples were removed from the rotor, dried for 1 day at ambient conditions, and in order to distinguish the (otherwise white) leached layer from the remaining (white or very light coloured) coating, a thick blue line was drawn by a marker. The marker works as leached layer indicator because the capillary pressure ensures penetration as long as there are empty pores. The samples were cut in half and cast in paraffin, and the internal front of

the sample was planed off before total film thickness and leached layer thickness was established using light microscopy.

Antifouling efficiency

Laboratory antifouling assays were performed using barnacles. Barnacle larvae of the species *Balanus improvisus* were retrieved from the institution of Marine ecology at University of Gothenburg. The assay was performed in the coated Petri dishes prepared for release rate measurements (cf. above), and the method was partly adopted from that described by Rittschoff et al. (Rittschof et al. 1992). Ten ml of filtered seawater was used, and the test animals were transferred using a small cloth mounted on a stirring rod. Barnacle activity and death were evaluated after 24 and 48 hours, and the hydrogen peroxide concentration was measured using Merckoquant® equipment.

Antifouling potential under real-life conditions was established by immersing panels covered with experimental coating in seawater as described by Kiil et al. (2006). Polycarbonate fibre panels with a dimension of 10*20 cm were used. The panels had been primed with Hempels Hempatex high-build 4633 to improve adhesion strength between panel and experimental coating. The inorganic peroxide-based, experimental coatings were applied using 8 cm Dr Blake applicator with a gap of 350 µm. The (uncoated) edges of the panels were covered in commercial antifouling paint to prevent biofouling from developing from unprotected areas of the panels. The panels were mounted on a raft in Singapore (1^{o} 23’33 N, 103^{o} 58’ 34 E), where the conditions are tropic, and therefore provide maximal fouling stress on the coatings (Kiil et al. 2006). The water depth at the raft site was 3 meter, the salinity 3.2 to 3.5 % and seawater temperature varied between 32 and 35 °C.

The coatings based on fast polishing binder compositions were immersed for 40 weeks initiated in week 14 (April) 2007, and ended week 2 (January) 2008. The results from these coatings motivated the broader trial, which is why the intermediate and slow polishing coatings were only immersed for 2 months from week 48 (November) 2007 to week 3 (January) 2008. The intermediate and slow polishing coatings were immersed in the same depth, but pointing in separate directions.

The coatings were inspected every four weeks. They were graded visually based on the area of the experimental coating that was covered with fouling. A grade was given for each of the biofouling classes: slime, algae and animals. Photographs of the panels were taken to support the grades.

Results

Critical pigment volume concentration

The established oil absorption values and their level of confidence are provided in Table 3.2 together with the corresponding critical pigment volume concentrations (CPVC). Magnesium peroxide had the lowest oil absorption value and consequently the highest CPVC (56 vol %), followed by zinc peroxide (53 vol %), calcium peroxide (45 vol %), and strontium peroxide (39 vol %).

Particle size distribution

The results of the particle size distribution measurements are shown in Figure 3.1. The peroxides of magnesium, calcium, and strontium were all primarily smaller than 10 µm, but a tendency towards agglomeration was seen. Zinc peroxide however, was distributed between 1 and 20 µm with even larger agglomerates.

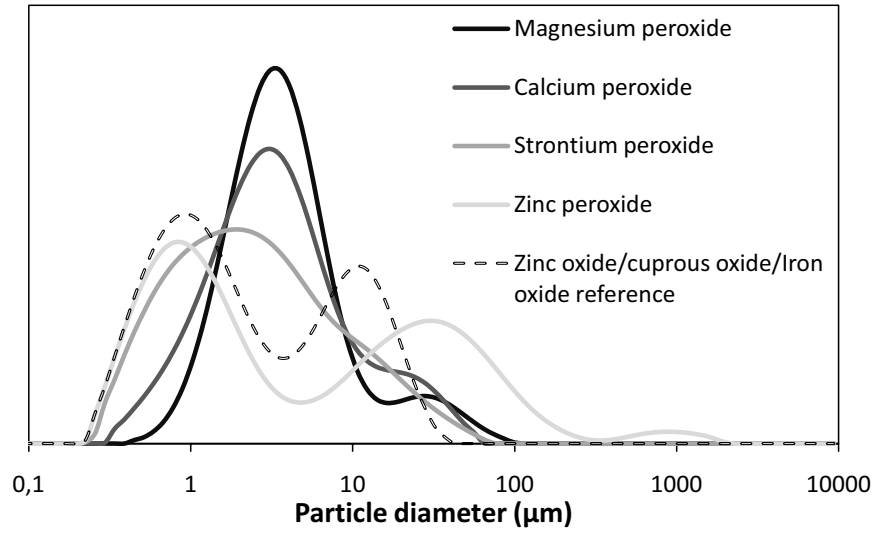

Figure 3.1: Particle size distribution (PSD) of the pigments. The distributions have been measured on dry powder. And the volume based distribution is shown.

Water immersion test

Strontium-, and calcium- peroxide-based coatings did generally take up more water than zinc peroxide-based coatings, and as can be seen in Figure 3.2, the weight gain of slow and fast polishing calcium peroxide coatings, and slow and intermediate polishing strontium peroxide coatings were extremely high. The weight increases of the two references included in the test are 20 and 12 wt% for #1 and #2 reference coatings respectively. It is therefore expected that the coating should not gain significantly more weight than 20% before the mechanical properties of the coating is compromised.

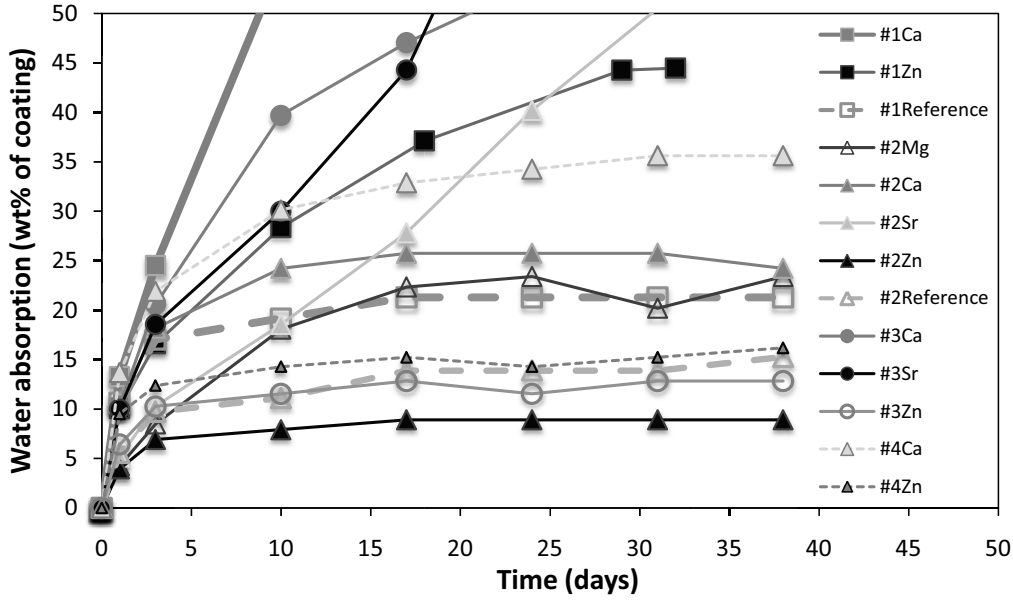

Figure 3.2: Water immersion of experimental coatings. See Table 3.3 for coating compositions.

Hydrogen peroxide release rates

The results of the Merckoquant® measurements of hydrogen peroxide accumulation in artificial seawater are provided in Figure 3.3. Though the colorimetric determination does not allow for great quantitative accuracy, the coatings containing SrO_2 and CaO_2, hydrogen peroxide showed easily identifiable accumulation. For these coatings, the release rates were established. For the intermediate polishing coatings, the release rates were 27 and 20 µg/cm2*d for calcium and

strontium respectively, and for the slow polishing coatings, the release rates were 29 and 19 for calcium and strontium respectively. The release rate measurements during the 11 days the experiment ran are shown in Figure 3.4. It is seen that the release rate for the intermediate polishing binders stabilised during the initial 10 days. For calcium peroxide, the steady-state release rate was 21µg/cm^2*d, and for strontium; 17µg/cm^2*d, but the release rates continued to decrease for the slow polishing coatings.

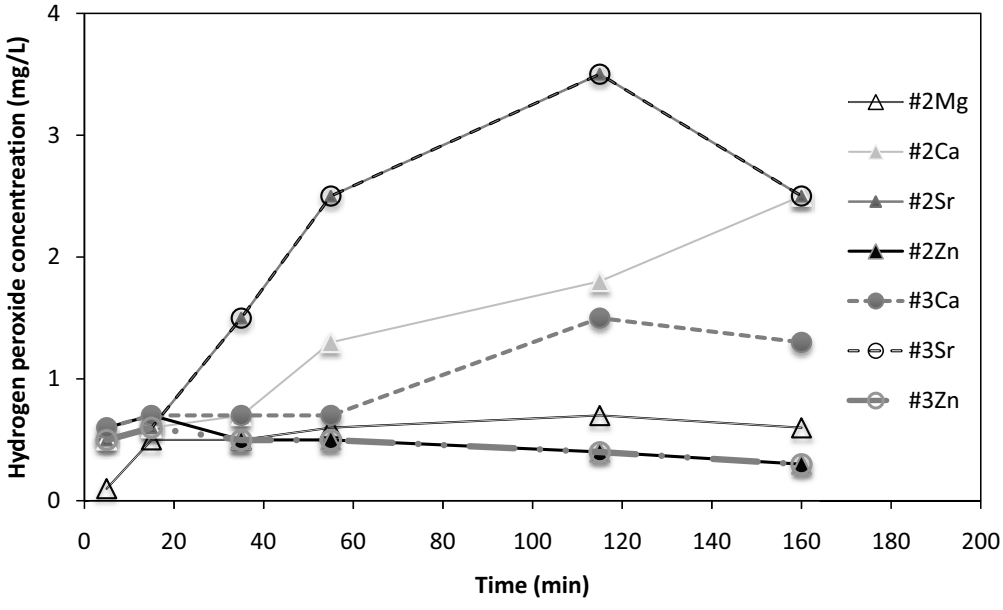

Figure 3.3: Accumulation of hydrogen peroxide in artificial seawater. Concentrations were measured using Merckoquant® equipment, after 5 days conditioning in artificial seawater. See Table 3.3 for coating compositions.

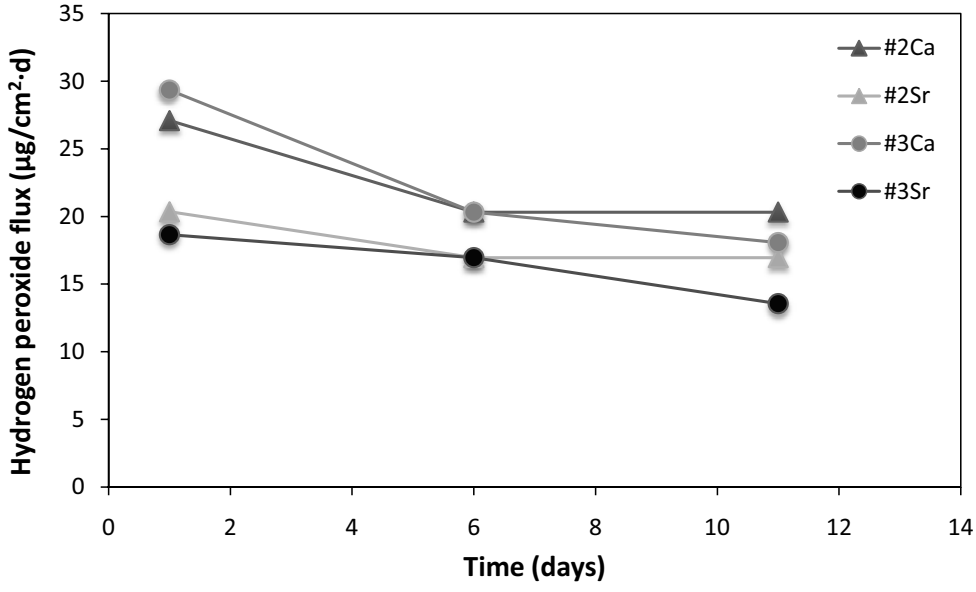

Figure 3.4: The development of release rates of the most potent hydrogen peroxide over the initial 12 days immersion in artificial seawater. See Table 3.3 for coating compositions.

Results for zinc peroxide and magnesium peroxide-based coatings have not been included as the measured hydrogen peroxide concentrations were too low for detection and did not deviate over time.

Table 3.4: Release rates and coefficients of determination for the experimental coatings. ID = Inconsitent data. BDL = below detection limit of hydrogen peroxide concentrations. See Table 3.3 for coating compositions.

	Release rate in Artificial seawater (µg/cm2·d)	Release rate in deionised water (µg/cm2·d)
#1Ca	Not measured	61±24*
#1Zn	Not measured	BDL
#2Mg	0.7±0.1	6±3
#2Ca	1.7±0.2	27±3
#2Sr	20±3	101±14
#2Zn	ID	ID
#3Ca	ID	15±1.6
#3Sr	ID	ID
#3Zn	ID	ID

* Measurements were performed with mercoquant (TM) sticks. The reported release rates is an average of three measurements at day 20, 27 and 32.

Spectrophotometric analysis of hydrogen peroxide allowed for lower detection limits. However, in artificial seawater, only the intermediate polishing strontium peroxide coating had a hydrogen peroxide accumulation, which was continously increasing. The accumulation of hydrogen

peroxide in deionised water (and in artificial seawater where possible) were converted to release rates. These can be seen in Table 3.4. It is seen that the potency of the peroxides generally follows the trend: Zn<Mg<Ca<Sr.

Polishing and leaching

The two fast polishing coatings (i.e. zinc peroxide and calcium peroxide) were swept off the substrate within three days of initiation of the rotary experiment, but the remaining coatings remained intact during the extent of the experiment (8 weeks). Microscopy of the samples revealed that only the coatings based on the intermediate polishing binder system polished. Figure 3.5 shows the development of coating and leached layer thicknesses for the coatings where polishing was identified. The error bars indicate the confidence levels, which were calculated by measuring the thickness at several points of one sample. It can be seen that all the coatings that polished also swelled initially following immersion. The slopes for where the total coating thickness decreases were established, and the rate of polishing calculated. The results are presented in Table 3.5. It is seen that the polishing rate ranges from 28 to 112 µm/month following the order Zn<Sr<Mg<Ca.

Table 3.5: Polishing rate and final leached layer thickness of the coatings based on intermediate polishing binder composition. The uncertainties indicate the 95% confidence level of the values.

	Polishing rate (µm/month)	Number of measurements during polishing
#2Mg	46 ±4	2
#2Ca	112±4	2
#2Sr	39±3	3
#4Ca	28±2	4
#2reference	45±6	4

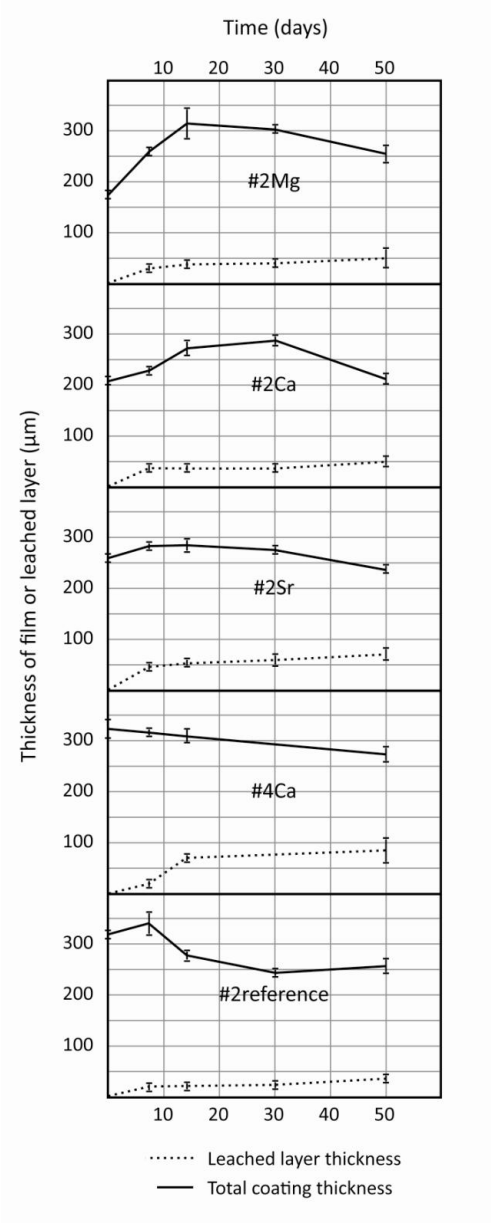

Figure 3.5: Polishing and leaching performance of experimental coatings based on the intermediate polishing binder system. Bars indicate the confidence level of thickness of one sample.

The coatings formulated with slow polishing binder, and those containing zinc peroxide, did not polish at all during the 50 days the experiment ran. They did not swell much either, so only the

development of the leached layer is presented in Figure 3.6. It is seen that the leached layer thickness for the coatings generally continue to increase. However the leached layer thickness for #3Sr seems to level off at a value around 70 µm.

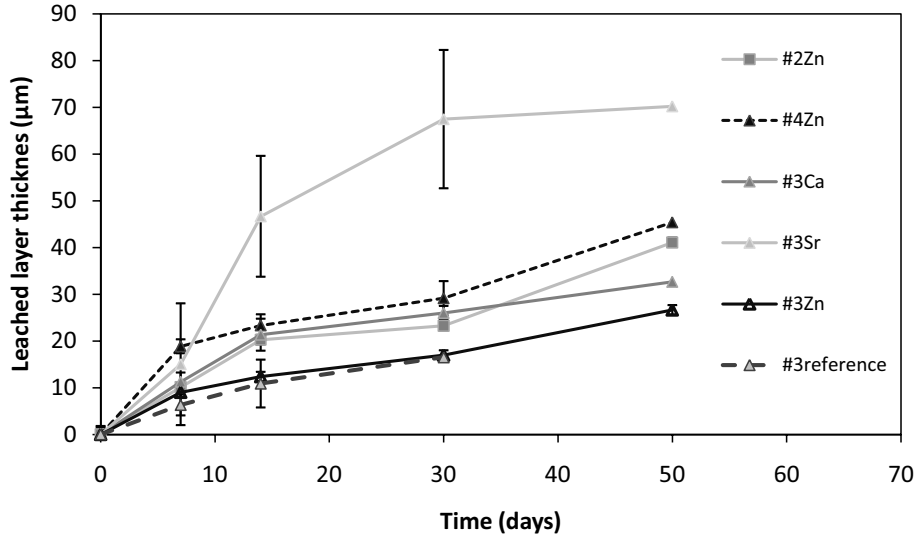

Figure 3.6: Leaching of experimental coatings. The coatings presented in the figure did not polish during immersion on the rotary system, and therefore only leaching data is provided.

Antifouling efficiency

Laboratory experiment

The result of barnacle experiments in laboratory assays are shown in Table 3.6. From the table, it can be seen that all the coatings affect the barnacle cyprids. However, it is also seen that a fair amount of hydrogen peroxide can be found in the water at the time of inspection.

Table 3.6: Antifouling performance of coated Petri dishes on barnacle cyprids in laboratory assay. See Table 3.3 for coating compositions.

Coating	Active barnacles after 1 Hour (amount)	Active barnacles after 24 Hours (%)	Active barnacles after 48 Hours (%)	Hydrogen peroxide concentration after 24 hours
Blank glass Petri dish	25-30	~100%	~100%	0
#1Ca	50-60	~2%	0%	*
#1Zn	30-35	~0%	0%	*
#2Mg	25-30	~25%	0%	~0.5
#2Ca	25-33	~5%	0%	2-5 ppm
#2Sr	25-30	~5%	0%	2-5 ppm
#2Zn	25-30	~25%	0%	<0.5 ppm
#3Ca	25-30	~5%	0%	0.5-2 ppm
#3Sr	25-30	~5%	0%	2 – 5 ppm
#3Zn	25-30	~25%	0%	<0.5 ppm

* Not measured.

Seawater experiment

The evaluation of the antifouling ability after four and eight weeks immersion of the intermediate and slow polishing binder systems are presented in Table 3.7. It is seen that the experimental coatings all foul to a great extent during 8 weeks immersion. The area based evaluations of the biofouling-type present on the different coatings after 8 weeks, are presented in Figure 3.7. The figure shows that the biofouling pattern is almost identical between all the inorganic peroxide based coatings.

Table 3.7: The grade given by the panel inspector after four and 8 weeks immersion to seawater in Singapore. See Table 3.3 for coating compositions.

Coating	Grade after 4 weeks	Grade after 8 weeks	Direction
Blank	Good	Fair	North
#1reference	Excellent	Excellent	South
#2Mg	Excellent	Poor	North
#2Ca	Good	Poor	North
#2Sr	Good	Poor	South
#2Zn	Excellent	Poor	North
#2reference	Excellent	Excellent	South
#3Ca	Excellent	Poor	North
#3Sr	Good	Poor	South
#3Zn	Excellent	Poor	North
#3reference	Excellent	Excellent	South
#4Ca	Excellent	Poor	North
#4Zn	Good	Poor	North
Commercial reference	Excellent	Excellent	South

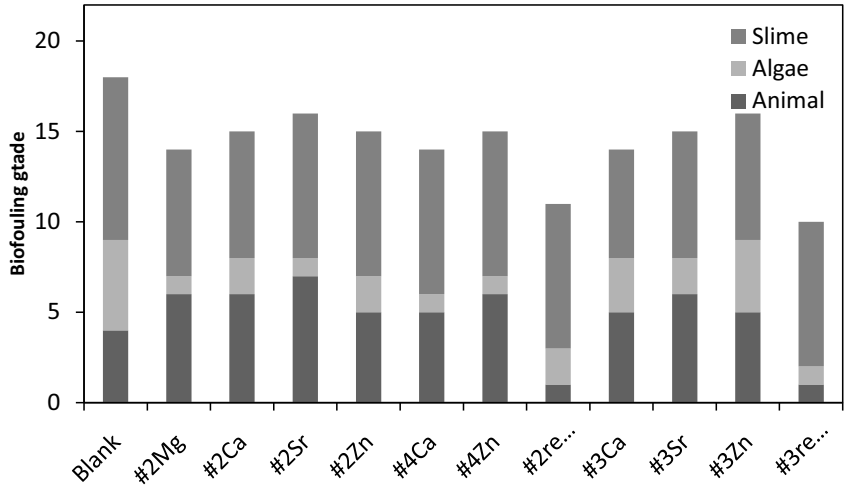

Figure 3.7: Area-based evaluation of the biofouling types settled on the static raft panels.

In Figure 3.8, the grade for the fast polishing coatings after 8 weeks immersion in Singaporean seawater is presented. The figure serves as comparison to the seawater experiment of the intermediate and slow polishing coatings.

The development of animal biofouling on the coatings over the 40 weeks the experiment ran is presented in Figure 3.9, and pictures of the panels after 24 weeks immersion are presented in Figure 3.10. It is seen that the fast polishing calcium peroxide coating was no better than blank acrylic panels after the initial weeks. However, the fast polishing zinc peroxide coating was performing better than the fast polishing zinc oxide-based reference. After 40 weeks immersion, the calcium peroxide based coating was completely lost. From pictures of the panels taken during the experiment, it was concluded that the coating was lost at some point between week 8 and 16 of the immersion. Therefore, only the fast polishing zinc peroxide coating performed satisfaction in the seawater experiment.

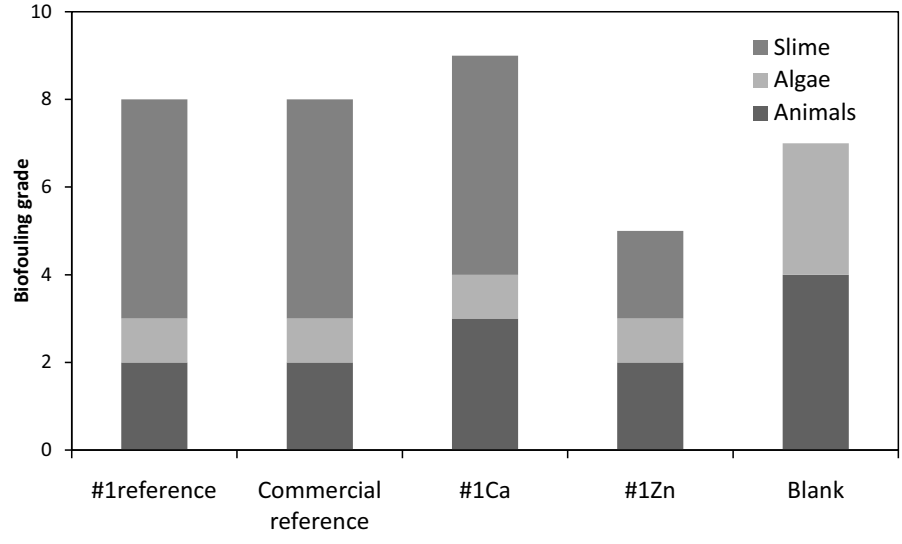

Figure 3.8: Area-based biofouling grade for fast polishing coatings after 8 weeks immersion in seawater in Singapore.

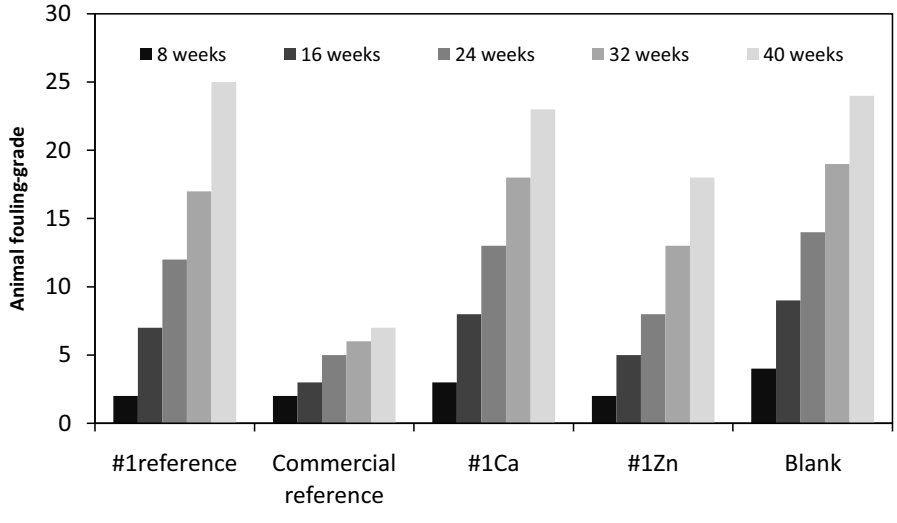

Figure 3.9: Development of area-based animal biofouling grade on fast polishing experimental coatings. All, but the common antifouling reference had completely polished during 8 months static immersion in seawater. For zinc oxide and zinc peroxide based coatings, this corresponds to approximately 25µm/month.

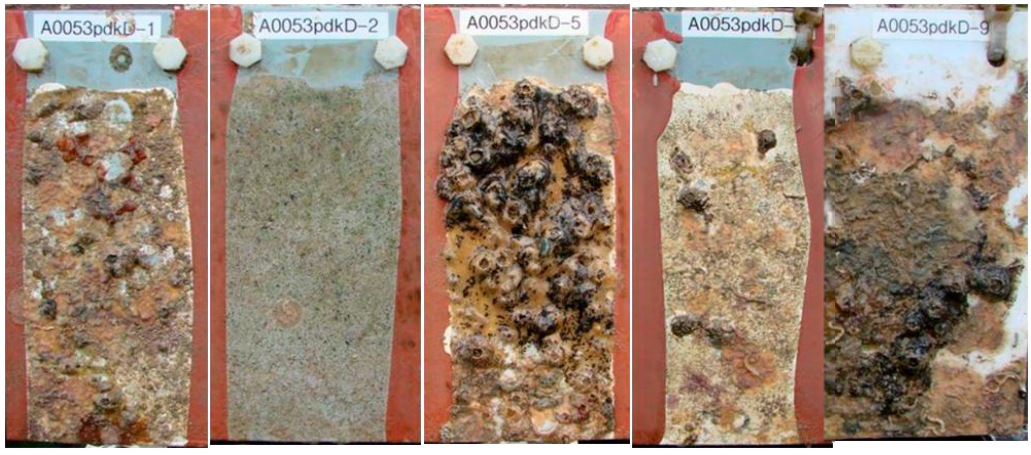

Figure 3.10: Pictures of panels for seawater experiment after 24 weeks immersion in Singapore. From left to right: Zinc oxide based reference; common antifouling reference; calcium peroxide-based fast polishing coating; zinc peroxide containing fast polishing coating; acrylic blank panel.

Discussion

The reaction between inorganic peroxides and water tend to result in oxygen in addition to hydrogen peroxide (Steriner and 2001). If all the available content of peroxide is liberated as hydrogen peroxide, a theoretical hydrogen peroxide yield per volume unit of pigment can be calculated from the densities, molar masses, and content of peroxide of the pigments. Such figures are presented in Table 3.2; they serve to compare the potency of the peroxides as raw materials. It is seen that strontium peroxide in its pure form is the most peroxide-liberating of the pigments. This is also the case when the values are modified using the CPVC values. These considerations comply with the results obtained here (cf. Table 3.4).

Linseed oil is susceptible to oxidation, and utilising linseed oil in measurements of oil absorption of oxidative species may cause oxidation of the linseed oil. This means that the oil absorption measured can have been that of the reduced material (i.e. oxides and hydroxides) and not the peroxide content.

Measurements of particle size distributions were performed several months after paint production, and it is seen that the pigments tend to agglomerate during storage. However, this tendency is most pronounced for zinc peroxide. The reference included in the figure contains small zinc oxide particles and larger cuprous oxide particles as well as some iron oxide. It should be noted

that homogeneous films were obtained from all the pigments when prepared within short time of dispersion. Stable coatings may therefore be obtainable if dispersion agents are used.

In the water immersion test, the faster polishing binder showed high weight gains. This indicate higher water absorbtion, which is due to an increased amount of hydrophillic binder constituents added to increase rate of polishing. However, the weight increase of the panels is not exclusively caused by absorption of water, but by the summarised effect of absorbed water, precipitates, and material loss from coating to surroundings. Precipitates were identified on all coatings containing magnesium, calcium and strontium peroxides. These precipitates were identified as carbonates by use of Fourier transform infrared spectroscopy. However, carbonates of the compounds are at most 47, 84, and 23 % heavier than the commercial peroxide products for calcium, magnesium and strontium, respectively, so carbonates cannot account for all the weight gained during the water immersion experiment.

The release rates of hydrogen peroxide from the coatings were established by measuring the accumulation in a stationary water phase over time. However, the accumulation measured equals the released amount of hydrogen peroxide subtracted the amount that had decomposed. Therefore the results are only indicative of the actual release rates. In Table 3.4, a difference in release rates is identified when artificial seawater is substituted for deionised water. This effect can be caused by a difference in the rate of decomposition of hydrogen peroxide, but it can also be a result of increased water activity of deionised water. This is because water absorption of coatings is known to be dependent on the salinity of the water; the higher salinity, the lower water absorption (Eul et al. 2001). Another odd observation is the tendency of the hydrogen peroxide concentration to decrease, which may be caused by a slower release, or a faster decomposition of hydrogen peroxide. For the former, clotting of the pores would limit the release rate during water immersion, and for the latter, the alkaline products developed during the reaction between water and the inorganic peroxide can increase the rate of hydrogen peroxide decomposition. Hydrogen peroxide is more rapidly decomposed in alkaline environments (Wicks 2002). However, it has not been possible to identify any pH change when tried, and Kiil et al. (2002) report that even a small pH increase in the seawater surrounding an antifouling coating cannot be obtained by the pigmentation of the coating.

The faster polishing coatings were not stable on the rotor. This means that no polishing and leaching-data is available for the well performing coating, but it also indicates limited mechanical integrity for these coatings. Improving the mechanical properties of the coatings is a necessity in order to obtain a coating that can be thoroughly tested and ultimately obtain a coating to be

commercialised. However, modifying the binder in order to provide more mechanical stable coatings may counteract the antifouling properties reported in this paper, by lowering polishing rate.

Whereas magnesium-, calcium- and strontium peroxide ultimately gives alkaline by-products (group II metals), zinc peroxide is the only pigment, investigated in this paper, which gives amphoteric by-products when reacted with water. This means that the hydrogen peroxide release is expected to be more stable when the precursor peroxide is zinc-based. In Figure 3.5, polishing is easily identified. However, the initial swelling of the coatings resulted in only a few measurements performed where polishing could be seen. Therefore, in order to establish the exact rate of polishing for the experimental coatings in question, further studies are required. Comparing Figure 3.5 and Figure 3.6 shows that wheares the leached layers were levelling off resembling steady state for the polishing coatings, they kept increasing for the coatings that did not polish. This complies with the theory describing insoluble matrix coatings (cf. the introductory section).

The grades that represent the antifouling potential of the coatings are given based on the area of the coated panel covered with the type of biofouling in question. Therefore a high value of animal biofouling can automatically mean a low value in algae and slime biofouling. When evaluating the results, the primary focus should therefore be on the antifouling potential towards animals.

An unintended consequence of the antifouling trials described in this paper is the elucidation of the gap between laboratory antifouling assays and real seawater trials. It is evident from Table 3.6, that all the experimental coatings are very potently killing barnacle larvae. However, when looking at the seawater trials (Table 3.7), the inorganic peroxides turn out to provide insufficient biofouling protection. After being immersed for 8 weeks, the animals were removed, and a cross section of the coatings was inspected in microscope.

Table 3.8: Microscopic inspections of raft panels after ended immersion.

Coating	Conclusion
#2Mg	Coating completely missing
#2Ca	Complete leaching of film
#2Sr	Coating completely missing
#2Zn	Inhomogeneous leaching depths and cracks in the film
#4Ca	Complete leaching
#4Zn	Inhomogeneous leaching depths and cracks in the film
#3Ca	Inhomogeneous leaching depths
#3Sr	Complete leaching
#3Zn	No leaching

Table 3.8 shows the measured polishing and leaching rates. It is seen that the experimental panels can be grouped in four:

1) Panels with completely leached coatings.
2) Panels where no leaching had yet occurred.
3) Panels with complete loss of coatings.
4) Panels where there were still inorganic peroxide-containing coatings left.

It is evident that group 1 is a consequence of very reactive pigments enclosed in binders that allow a significant amount of water into the film, and group 2 is due to inactive pigment in coatings that take up little water. Group 3 on the other hand is not as easily accounted for. One explanation can be that the oxygen produced as an alternative to, or a consequence of, hydrogen peroxide (Steiner and Eul 2001) builds up within the pores of the leached layer. In fact bubbles were identified on coatings containing calcium and strontium peroxide in laboratory experiments. Production of gaseous compounds within the coating will stress the coating severely, and the internal stress may result in rupture of the coatings during the natural motion of the water outside.

Of the four groups listed above, only the fourth is of interest. These are the only panels, for which potential antifouling effects (or lack of same) can be ascribed to the active ingredient in the coatings. The group contains the three experimental coatings; intermediate polishing zinc peroxide, intermediate polishing high PVC zinc peroxide, and slow polishing calcium peroxide. By comparing the original thickness of the slow polishing CaO_2 coating (found under the commercial antifouling coating applied on the edges) with the thickness after immersion, it was seen that the coating had not polished. The leached layer was identified using a marker, but the colour of the marker might not have penetrated the surface of the coating where the biofouling had been intense. If the marker is used as leached layer cursor, there had been leaching in a depth ranging between 0 and 50 µm. The leached layer depth that would be expected based on the experience from the rotary experiment extrapolated to 50 days immersion corresponds to between 40 and 50 µm (cf. Figure 3.6). The accumulation of slime and biofouling in general influences the rate of leaching (Yebra et al. 2006), which can explain the very inhomogeneous leaching depths found in all the intact coatings. Considering the two zinc peroxide coatings, cracking is seen in both of them, and where biofouling animals had been removed; the coating was significantly thinner. Also in the case of the zinc peroxide coatings, the leached layer thickness varies and cannot be measured accurately. However, in both cases the maximum identified leached layer depth is 60 µm.

From Figure 3.9, it is evident that the zinc peroxide based coating outperforms the zinc oxide-based reference. Because the film will release Zn^{2+} as well as hydrogen peroxide the reference containing zinc oxide was included. The reference also released Zn^{2+}, and therefore comparison between them gives the effect of hydrogen peroxide. Both coatings had polished almost completely off during the 8 months static immersion in seawater. Therefore, total amount of zinc in the coatings was used to estimate the average release rates of zinc and hydrogen peroxide during the period of testing. Calculated as release rates, the zinc peroxide based coating released zinc with an average rate of 0.44 µmoles/cm^2*d, and hydrogen peroxide was release with a rate of 0.22 µmoles/cm2*d. In comparison, the zinc oxide based reference had released zinc with an average rate of 0.51 µmoles/cm^2*d. It is therefore reasonable to conclude that the release of hydrogen peroxide is responsible for the reduction in fouling seen on the peroxide-based coating compared to the zinc oxide reference.

All the peroxides are strong oxidisers that may cause fire on contact with combustible materials (Eul et al. 2001). Solvent borne coatings will always contain combustible materials, and water will react with the pigment. Therefore the production of an inorganic peroxide based coating can turn out to be a fire hazard. Ketones react with peroxides to give explosive compounds (acetone peroxide as the best known example). To produce coatings based on inorganic peroxides, potential reducing compounds should be omitted, and the extent of the fire hazard should be uncovered before experimenting further.

Conclusions

This paper establishes the applicability of inorganic peroxides in antifouling coatings. It is documented that all the pigments leach from the coating in a controlled manner under laboratory conditions. Polishing of inorganic peroxide-based coatings is also documented.

Under real-life conditions, immersed in the sea, calcium-, magnesium-, and strontium peroxide fail to retain the mechanical properties required of an antifouling coating. As active antifouling ingredient, only zinc peroxide provides suitable mechanical properties for long term seawater exposure, as well as sufficiently potent antifouling properties, and only when a fast polishing binder system is applied. Zinc peroxide in a fast polishing coating outperforms a zinc oxide-based reference in direct comparison, and under the circumstances described in this paper. The improved antifouling ability of the zinc peroxide-based coating is shown to be caused by the peroxide content of the coating. It is also clear from the results provided here that zinc peroxide

cannot compete with the more potent cuprous oxide and co-biocides that are commonly used today. Nor is it believed that the gap between these two pigments can be filled by optimisation of the zinc peroxide based coating. It is therefore not likely that a zinc peroxide based coating will exceed a service life of 1 year.

The commercial potential of zinc peroxide based antifouling coatings should therefore be estimated based on an evaluation of antifouling potential and security risks. If decided so, further development is required before a safe to handle, mechanically stable, antifouling efficient coating based on zinc peroxide is obtained.

References

Asghari A, Farrah S R. 1993. Inactivation of bacteria by solids coated with magnesium peroxide. Envion. Sci. Health **A28**. p 779-793.

Elzanowska H, Wolcott R G, Hannum D M, Hurst J K. 1995. Bactericidal properties of hydrogen peroxide and copper or iron-containing complex ions in relation to leukocyte function. Free radical & medicine **18**. p 437-449.

Eul W, Moeller A, Steiner N. 2001. Hydrogen Peroxide. Kirk-Othmer Encyclopedia of Chemical Technology. p 1-53.

Greenfield D, Cantlebury D. 2000. The protective action of organic coatings on steel: A review. Journal of corrosion science and engineering **3**(5). p 146-158.

Ikuta S, Ichikawa S, Wakao Y, Nishimura K, Yasunaga T. 1988. Inorganic Peroxides for heat exchanger biofouling control. Jt. ASME/IEEE power generation conference. Philadelphia, Pennsylvania, September 1988.

Kiil S, Dam-Johansen K, Weinell C E, Pedersen M S. 2001. Analysis of self-polishing antifouling coatings using rotary experiments and mathematical modelling. Ind. Eng. Chem. Res. **40**. p 3906-3920.

Kiil S, Dam-Johansen K, Weinell C E, Pedersen, M. S. 2002. Seawater soluble pigments and their potenital use in self-polishing antiofuling coatings: a simulation-based screening tool. Progress in organic coatings **45**. p 423-434.

Kiil S, Weinell C E, Yebra D M, Dam-Johansen K. 2006. Marine biofouling protection: design of controlled release antifouling paints. In: Ng K M, Gani R, Dam-Johansen K (ed). Chemical product design; towards a perspective through case studies. 23IDBN-13: 978-0-444-52217-7. part II (7). Elsevier.

Mathyarasu J, Marutiamutiu S, Muralidharan S, Meenakshisundaram R, Rengaswamy N S. 1997. Evaluation of peroxide based biocides for inhibition and biocidal efficiencies. Bulletin of Electrochemistry **13**. p 289-293.

Minoru K, Shunkai M, Katsumi K. 1988. Antifouling composition. Japanese patent 01-213372.

Minuro K, Shunkai M, Katsumi K.. 1989. Coatings containing non-metallic antifouling agent releasable compounds. Japanese patent application 64-61404.

Morris R S, Walsh I V. 1996. Zinc oxide photoactive antifoulant material. US patent 5,916,947.

Neale M. 2003. The biocidal products directive- industry concerns. Pesticides outlook **14**. p 71-73.

Nishimura K, Yasunaga T, Ichikawa S, Wakao Y. 1988. Development of a new antifouling method for a marine cooling water system **99**. p 145-150.

Olsen S M, Pedersen L T, Kiil S, Hermann M H, Dam-Johansen K. 2007. Enzyme-based antifouling: a review. Biofouling **23**. p 369-383.

Rittschof D, Clare A S, Gerhart D J, Mary S A, Bonaventura J. 1992. Barnacle in vitro assays for biologically active substances: toxicity and settlement inhibition assays using mass cultured Balanus amphitrite amphitrite Darwin. Biofouling **6**(2). p 115-122.

Savary B, Hicks K, O'Connor J. 2001. Hexose oxidase from Chondrus crispus: improved purification using perfusion chromatography. Enzyme and Microbial Technology **29**. p 42-51.

Steiner N, Eul W. 2001. Peroxides and peroxide compounds. Inorganic peroxides. Kirk-Othmer Encyklopedia of chemical technology **18**. p 1-35.

Waite A J, Bonner J S, Autenrieth R. 1999. Kintetics and stoichiometry of oxygen release from solid peroxides. Environmental engineering science **16**. p 187-199.

White D M, Irvine R L, Woolard C R. 1998. The use of solid peroxides to stimulate growth of aerobic microbes in tundra. Journal of hazardous materials **57**. p 71-78.

Wicks Z W. 2002. Coatings. Kirk-Othmer Encyclopedia of Chemical Technology **7**. p 77-150.

Yebra D M, Kiil S, Weinell C E, Dam-Johansen K. 2003. Review: Antifouling technology- Past, present and future steps towards efficient and environmentally friendly antifouling coatings. Progress in organic coatings **50**. p 75-104.
Yebra D M, Kiil S, Weinell C E, Dam-Johansen K. 2006. Mini-review: Presence and effects of marine microbial biofilms on biocide-based antifouling coatings. Biofouling **22**. p 33-41.

4. Chapter four - Replacement of traditional seawater-soluble pigments by starch and hydrolytic enzymes

This chapter concerns the development of a polishing antifouling coating based on starch as the active ingredient. The slow degradation of water-insoluble starch into water soluble glucose by the hydrolytic enzyme, glucoamylase is used to mimic the working mechanism of polishing antifouling coatings based on water soluble metal oxides. Initially, the working mechanism of polishing antifouling coatings is described. This is followed by a description of the tests performed to select the optimal starch type for use as antifouling coating ingredient. Coatings formulated containing starch and glucoamylase has been tested for common coating characteristics, and rate of polishing and leaching has been obtained.

The content of this chapter is intended for publishing in the journal of coating technology and research during 2009. The title of the paper will be 'Replacement of traditional seawater-soluble pigments by starch and hydrolytic enzymes' (Authors: Olsen S M, Pedersen L T, Kristensen J B, Dam-Johansen K, Kiil S).

List of symbols

Ci	Starch type number i originating from corn
C_s	Seawater solubility in mol/m^3
M	Molar mass kg/mol
Ri	Starch type number i originating from rice
T	Starch type originating from Tapioca
α	Seawater solubility dimensionless
ρ	Density kg/m^3

Abbreviations

CPVC	Critical pigment volume concentration in vol%
DFT	Dry film thickness in µm
LLT	Leached layer thickness in µm
OA	Oil absorption
PSD	Particle size distribution
PVC	Pigment volume concentration in vol%

Background

Working mechanism of antifouling coatings

Most antifouling coatings work by releasing biocides (seawater-soluble pigments and organic compounds) into seawater. This leaves behind a leached layer of biocide-depleted coating. If the thickness of the leached layer is continuously increasing to prohibitive values, the diffusion resistance of the dissolved biocidal compounds increases, and for most compounds this means that the flux of biocide at the interface between coating and seawater decreases to inefficient levels. This limits the service life of a coating, though it still contains biocide. The leached layer thickness should therefore remain constant and low (preferably 5-20 µm), and this is achieved by a steady erosion of the outermost layer of the leached binder system. This mechanism is called polishing, and is a requirement when developing new chemically active antifouling coatings. Figure 4.1 shows the mechanisms involved in polishing of an antifouling coating, dividing the process into three steps. 1: A freshly immersed antifouling coating will leach seawater-soluble pigments into the sea. 2: Seawater-filled pores left behind by the seawater-soluble pigments constitute the leached layer of the coating. The surface area of the water-binder interface is increased by formation of the leached layer, which allows for more water-binder interactions. 3: As a result the outermost layer of the binder is released into seawater.

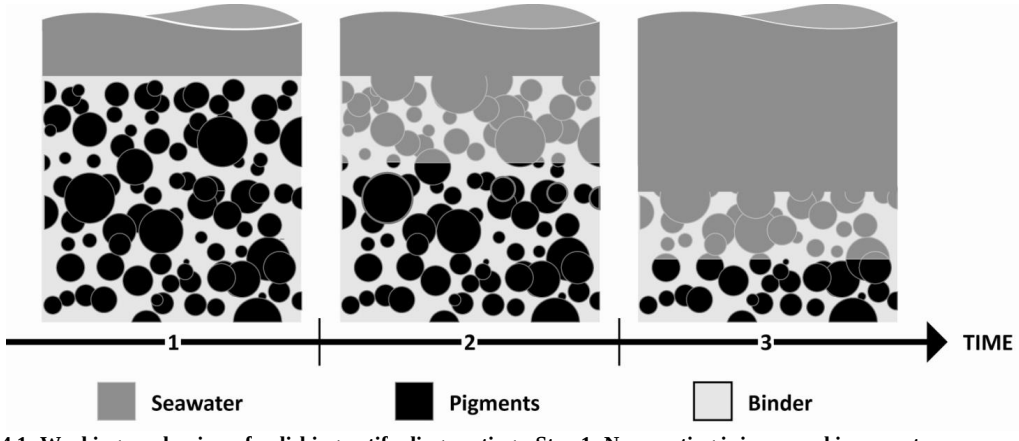

Figure 4.1: Working mechanism of polishing antifouling coatings. Step 1: New coating is immersed in seawater. Step 2: The water soluble pigments dissolve in seawater leaving behind a leached layer of seawater-filled pores. Step 3: The surface area of the leached layer accelerate the seawater reaction with the binder. Polishing is the combined result of chemical reactions and friction force from moving seawater (or vessel). For a properly working polishing antifouling coating, the leached layer thickness remains small and constant after a few weeks seawater exposure (Kiil et al. 2001).

Antifouling binder compositions

Polishing antifouling coatings can be divided into two types depending on the release mechanism of the binder material from the coating, soluble and insoluble matrix coatings. In soluble matrix coatings, the binder contains slightly soluble parts. When in contact with seawater, the soluble pigment will generally dissolve faster than the soluble binders, thereby forming a leached layer from which the soluble binder parts are released. The pigments continuously dissolve faster than the binder material, so the leached layer grows in thickness over time, and eventually this will cause ineffectiveness of the biocide (Yebra et al. 2006a). Soluble binder coatings contain a soluble binder matrix, and when rosin is used as soluble binder constituent, Ca^{2+} and Mg^{2+} from seawater may cause precipitation of salts in the leached layer (Yebra et al. 2005). These must be physically removed by motion of the water, and therefore soluble matrix coatings are leaching considerably less biocide, when the ship is not in motion (Yebra et al. 2006a). In general, adequate biocide release rates are obtained by a suitable matrix dissolution or reaction. Therefore, antifouling can in many cases be promoted by pigments or binders that increase the polishing rate without being biologically active. Relatively thick coatings (100-300 μm) are necessary to retain antifouling effect for longer periods (Yebra et al. 2006a).

So-called self-polishing coatings rely on a chemical reaction between seawater and the binder material (e.g. the previously widely used tributyltin (TBT) methacrylate methylmethacrylate copolymers). In TBT-based coatings, the mode of action relies on hydrolysis of the ester bond linking the organotin to the acrylic backbone. This unleashes the biocide, but it also alters the physical properties of the acrylic backbone adding hydrophilicity and brittleness to the polymer, and when a suitable amount of ester groups have been hydrolysed, seawater erodes the outer layer of the coating and a fresh layer is exposed. In this manner hydrolysis of the ester bond controls both the release of biocide and the polishing of the coating. Seawater-soluble antifouling pigments are also added to these coatings to increase the antifouling effect, and the surface area of the binder. The steady-state thickness of the leached layer should be constant in most chemically reacting antifouling coatings (Yebra et al. 2006a).

Seawater-soluble pigments

Polishing of the binder is caused by chemical or physical interaction between binder and seawater, and the surface area of the binder is therefore one factor affecting the rate of polishing (Kiil et al.

2001; Kiil et al. 2002a). The leached layer effectively increases the surface area of the binder-water interface. As the specific surface area of the leached layer is determined by the soluble pigment that initially resided in the now empty pores, several pigment properties influence the polishing rate. Shape, particle size distribution, and rate of dissolution are pigment-parameters affecting the rate of polishing, and pigment volume concentration (PVC) is a pigment related coating parameter that can also be used to modify polishing rate (Kiil et al. 2002b).

The influence of pigments on polishing has been addressed by means of mathematical models and experiments (Yebra et al. 2006a; Kiil et al. 2001; Kiil et al. 2002a; Kiil et al. 2002b; Somasekharan and Ubrammanian 1980). For self-polishing TBT-based coatings, no polishing occurs if the coatings do not contain water-soluble particles such as Cu_2O and/or ZnO (Kiil et al. 2001), and polishing rate is increased with decreasing particle sizes, and increasing PVC values (Kiil et al. 2002b). In Kiil et al. (2002a), a mathematical model is used to screen for potential substitutes for cuprous oxide from a leaching and polishing point of view (i.e. not antifouling effect). According to the model, the dimensionless seawater solubility of the pigment, α ($\alpha = M \cdot C_s/\rho$) should lie between 10^{-6} to 10^{-8} in order to achieve polishing rates comparable to that obtained using cuprous oxide. As shown by Kiil et al. (2002a), the dimensionless solubility of most solids is much higher than 10^{-6}, and of the compounds screened in the paper, primarily very toxic heavy metal salts have suitable solubility values.

For soluble matrix antifouling coatings, polishing rate is increasing with decreasing amount of insoluble binder; decreasing particle size, and increasing PVC of seawater-soluble pigments (Yebra et al. 2006a). Mechanical stability of the coating is improved by adding insoluble pigments and binder constituents to the coating (Yebra et al. 2006a).

Strategy of investigation

The aim of this work has been to substitute commonly applied seawater-soluble antifouling coating-pigments for starch and starch degrading enzymes (glucoamylase). The starch is therefore added as an enzyme mediated water-soluble pigment, and successful implementation will provide an antifouling coating that can drive the release of an active antifouling biocide without the use of large quantities of heavy metal salts or oxides. The enzymes are only present in the coating in limited quantities, and only to facilitate degradation of the starch pigment. It has no antifouling active properties nor is it expected to reach the biofouling animals in substantial quantities.

Starch has, as a new coating ingredient, been evaluated for applicability in antifouling coatings. The best starch-type has been identified amongst 14 different starches obtained from rice, corn and tapioca. Formulated coatings containing the novel pigment (spray dried starch with glucoamylase at the surface) has been tested on a rotary set-up to monitor rate of polishing and leaching. It should be noted that neither starch, not glucoamylase should provide biofouling protection to the coating. These ingredients are only added in order to obtain polishing of the coating.

Table 4.1: Source, name and suppliers of the starches used. Whereas normal starch contains about 3/4 of the highly branched amylopectin, waxy starch is 100% amylopectin. Gelatinisation temperatures refer to the temperature at which the starches form a gel.

Name	Commercial name	Source	Supplier	Gelatinisation (°C)	Average equivalent spherical volume diameter (µm)
R1	Remy FG	Rice	Remy	65-73	2-8
R2	Remy B7	Rice	Remy	72	5
R3	Remygel 663	Rice	Remy	57	5
R4	Remy DR	Rice	Remy	77	5
R5	Remyline AX DR	Waxy rice	Remy	65-73	5
C1	C*gel 03401	Corn	Cargill	62-71	15
C2	Clearam MH 0500	Corn	Roquette	62-71	15
C3	Clearam MH 10 15	Corn	Roquette	62-71	15
C4	Clearam CI 30 00	Waxy corn	Roquette	62-71	15
C5	Clearam CI 10 00	Waxy corn	Roquette	62-71	15
C6	Clearam CH 15 05	Waxy corn	Roquette	62-71	15
C7	HI-CAT 21370	Waxy corn	Roquette	62-71	15
T1	Clearam TJ 2015	Tapioca	Roquette	59-70	20

Experimental

Starch

Starches were obtained from the companies Remy, Cargill, and Roquette. Table 4.1 shows the types of starches applied in the experiments covered by this paper. Also provided in the table are the supplier information on average particle sizes, and gelatinisation temperatures.

Particle size distribution

The particle size distributions of commercially obtained starches were measured using a Malvern Mastersizer 2000 from Malvern Instruments, and a Hydro 2000G sample disperser. The measurements were done on slurries of starch in ethanol, to avoid dissolution of water-soluble parts.

To establish the particle size distribution of the most compatible starch type (i.e. C1) after it had undergone coating production, particle size distribution of a full coating system was obtained. These measurements were done in xylene, and a few drops of the liquid paint were dispersed in commercial grade xylene, and particle size distribution was measured directly on the slurry.

Critical pigment volume concentration

The critical pigment volume concentration (CPVC) was estimated by measuring the oil absorption (OA) according to the DS/EN ISO 787-5:1995 method. Equation (4.1) describes the relations between oil absorption and critical pigment volume concentration.

$$CPVC = \frac{1}{\frac{OA \cdot \rho(pigment)}{100 \cdot \rho(oil)} + 1} \tag{4.1}$$

The oil absorption was established using commercial grade linseed oil. Approximately one gram of powder was used, and linseed oil was added and mixed until a paste was achieved.

Water soluble content of starches

The water-soluble content of starch-based ingredients was determined gravimetrically. Starch was mixed in deionised water and stirred effectively. The slurry was then centrifuged at 15,000 rpm for ten minutes, and the dry matter content of the supernatant was determined gravimetrically.

Coating formulation

Previous studies have shown that starch compromises coating integrity (Olsen et al. 2008), and agents, such as fibres and insoluble pigments must be included in the coating to provide sufficient mechanical strength over time. It was concluded from the above described experiments, that the starch-type C1 was the most compatible antifouling coating ingredient (see result and discussion section), and therefore only this starch type is considered.

The glucoamylase described by Dunn-Coleman et al. (2006) was used in this experiment. Starch and glucoamylase were spray dried from water-based slurry. 75 g of starch was added to 500 mg of an enzyme solution of 0.6 Glucoaymlase units/g slurry (1 glucoamylase unit corresponds to the amount of enzyme needed to produce 1 g of glucose in 1 hour). The slurry was then spray dried. Air inlet temperature was 135 °C, and powder outlet temperature was 80 °C. At the spray nozzle, water cooling with 0 °C water was used. The apparatus used was a Mini Spray Dryer B-191. In the slurry phase and during spray drying, glucoamylase binds to the surface of the starch granules at its starch binding domain (hydrogen bonding and van der Waals' forces) (Sauer et al. 2000).

The binder constituents used to formulate the experimental coatings contained zinc resinate (produced from rosin (Cas No: 65997-06-0) and commercial grade zinc oxide), poly (vinyl methylether) (Lutonal M40, 45% from BASF AG), and acrylate (methyl methacrylate/n-butyl methacrylate/methacrylic acid terpolymer (molar ratio approximately 100:100:1) purchased as Degalan LP 64/12, from Rohm GMBH).

Zinc resinate was produced by adding a threefold molar excess of zinc oxide (technical grade) to highly hydrogenated rosin (CAS nr: 65 997-06-0) dissolved in xylene. After dissolving for 1 hour, the slurry was left for two days, until infrared spectroscopy revealed almost 100% yield. Zinc resinate was separated from the excess of zinc oxide by centrifuging for 2 hours at 3000 rpm.

Screening tests (not published) indicated that a suitable binder composition was roughly 60-70 vol% zinc resinate, 15-25 vol% acrylate, and 10-20 vol% poly(vinyl methyl ether), and four physically dissolving binder systems were formulated within these ranges. The compositions of these are provided in Table 4.2. For each binder composition, one experimental coating containing starch and glucoamylase and two references were made; one containing starch with no enzyme, and one containing the common antifouling coating pigments zinc oxide and cuprous oxide in a ratio 1/3. In addition to the experimental pigmentation, all the coatings contained 5 volume % fibres (Rock fibre MS603 from Brenntag Nordic), 5 volume % Iron(III)oxide (colour pigment, Micronox H from Promindsa), and water scavenger and wetting agents in low amounts.

Table 4.2: The composition of experimental coatings. The amounts are given as volume percentages of dry coating. A, B, C, D denotes the different binder compositions, S refers to a starch reference, SG, to the starch and glucoamylase containing experimental coating, and R to the cuprous oxide-based reference.

Binder system	Zinc resinate	Acrylate	Poly(vinyl methyl ether)	Iron oxide	Fibres	Coating terminology	Starch	Starch & Enzyme	Cu_2O	ZnO
A	39	15	6	5	5	AS	30			
						ASG		30		
						AR			22.5	7.5
B	39	12	9	5	5	BS	30			
						BSG		30		
						BR			22.5	7.5
C	36	15	9	5	5	CS	30			
						CSG		30		
						CR			22.5	7.5
D	36	12	12	5	5	DS	30			
						DSG		30		
						DR			22.5	7.5

Water immersion test

Model coatings, composed of 39 vol% zinc resinate, 26 vol% acrylate and 35 vol% starch, were applied on $5 \cdot 10$ cm^2 polycarbonate panels and the panels were immersed in tap water adjusted to 45 °C. The panels were monitored gravimetrically during five weeks immersion. Before weighing, excess water was gently wiped off the panels. A commercially available coating (Mille Xtra from Hempel A/S) was included in the test series as a reference.

Polishing and leaching

Polishing and leaching characteristics were measured using a rotary set-up similar to the one described by Kiil et al. (2001), except the temperature was kept around 35 ºC. The rotor was operated at 20 knots during the experiment. The pH was adjusted frequently to 8.2 using 1 M sodium hydroxide or 1 M hydrochloric acid.

Samples were prepared using overhead transparencies (3M PP2410) that had been primed using two-component (a polyamide adduct curing epoxy) Hempadur 4518 from Hempel A/S to improve adherence to the smooth transparency film. Coating samples were applied adjacent to each other using a Dr Blade applicator with a gap of 250 µm. After curing, the coated transparency was cut in strips of 2 cm resulting in samples of $1.5 \cdot 2$ cm^2. The strips were mounted on the rotor, and with frequencies of 14 to 30 days, samples were removed from the rotor, dried for three days at ambient conditions, and in order to distinguish the leached layer from the remaining coating (both

red due to the insoluble iron oxide pigment), a thick blue line was drawn with a marker. The marker works as leached layer indicator because the capillary pressure ensures penetration as far down as there are empty pores. The samples were cut in half and cast in paraffin, and the internal front of the sample was planed off before total film thickness and leached layer thickness was established using light microscopy (coating cross-section inspection).

Blister box

The coatings containing starch and glucoamylase (i.e. the coatings, ASG, BSG, CSG, and DSG) were tested for their tendency to blister according to ASTM D 4585. The coated surfaces of the panels were exposed to 40 ˚C saturated water vapour, at an angle of 60° to the horizontal, and the back of the panels exposed to room temperature. The coatings were inspected every second week, and potential blistering was evaluated according to ASTM D714.

Results

Particle size distribution

The particle size distribution of the starch types considered for use can be seen in Figure 4.2. It is seen that the particle size distribution of starch depends on the source of starch. Rice starches differ considerably from corn and tapioca starches that resemble each other very much. Considering the mean particle sizes (about 5 µm) provided by the suppliers (see Table 4.1), it can be concluded that the rice starches shown in Figure 4.2 have agglomerated. The applicability of rice starch as an antifouling coating ingredient is therefore dependent on whether the agglomerates can be broken down and stabilised during production of the coating (this was not investigated in this work).

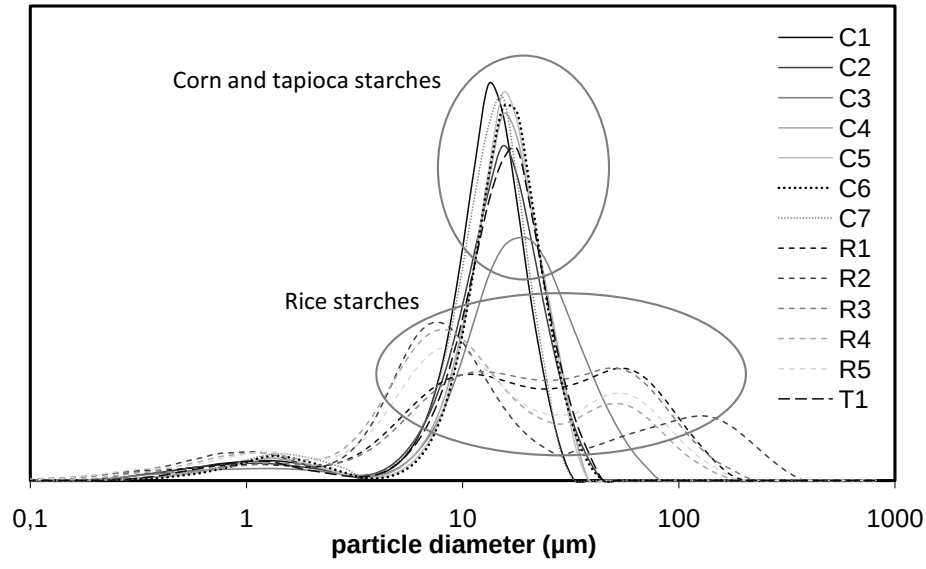

Figure 4.2: Particle size distributions (equivalent spherical volume diameter) of starch raw materials. See Table 4.1 for starch details.

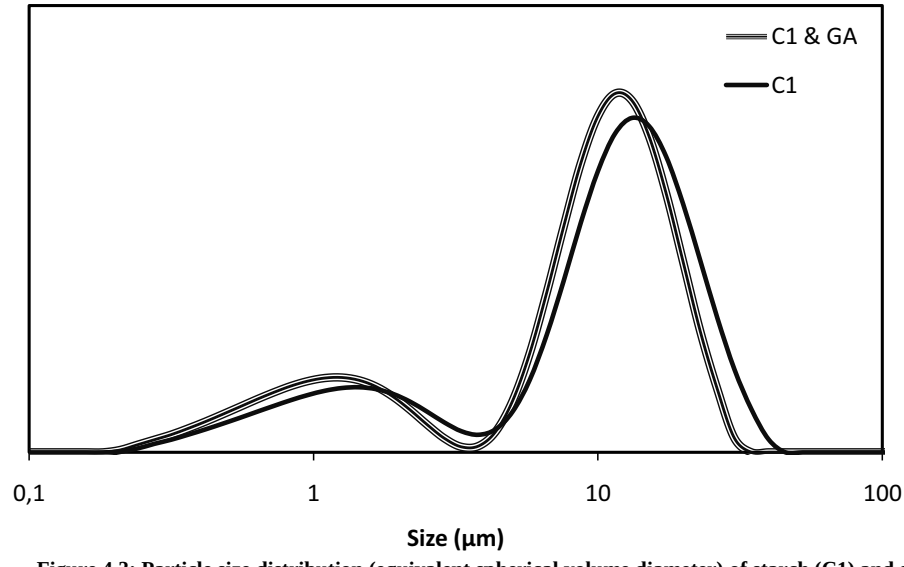

Figure 4.3: Particle size distribution (equivalent spherical volume diameter) of starch (C1) and starch and glucoamylase after spray drying. The measurements were done on formulated coatings containing iron(III)oxide, which accounts for the smaller particles around 1 µm in diameter.

Corn- and tapioca-starches are primarily distributed in the region between 10 µm and 20 µm, and antifouling coatings of dry film thicknesses between 200 and 300 µm can therefore contain these starches and still be considered homogeneous. Particle size distribution of starch, and starch and glucoamylase when in a formulated coating is shown in Figure 4.3. In the figure, iron(III)oxide pigments are seen in the region around 1 µm (Yebra et al. 2006b), and the starch peaks are unaltered compared to Figure 4.2, which shows the powder raw material as received by the supplier. The measurements were done several weeks after paint preparation, and it is therefore evident that corn starches when in a coating do not agglomerate, and stable liquid paints can be produced even with a considerable amount of starch in them.

Critical pigment volume concentration

The critical pigment volume concentration calculated from the oil absorptions measured, are shown in Figure 4.4. It is seen that all the rice starches, and the corn starch, C7, have critical PVC-values just below 50 vol%. The CPVC values for the remaining starch types all exceed 55 vol%. However, the values are adequately high for all the starch types to be formulated into the coatings described in Table 4.2.

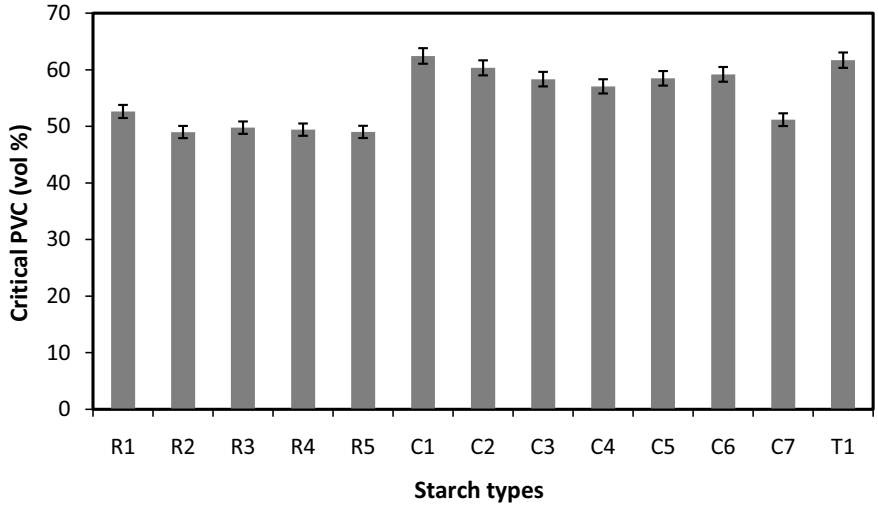

Figure 4.4: Critical pigment volume concentration (CPVC) of the starch types from Table 4.1. R= rice starches, C= corn starches, T= tapioca starch.

Water soluble content

The water soluble fraction of the different starch types is shown in Figure 4.5. It is an important requirement for an antifouling coating ingredient that it contains little or no water-soluble material. High quantities of very seawater-soluble material will draw water into the coating and cause blistering of the film. It is seen that corn starches generally contain significantly lower amounts of water-soluble material than do rice starches. Especially the starch types C1 and T1 have low amounts of water soluble contaminants; 0.12 ± 0.01 wt% and 0.13 ± 0.04 wt% respectively.

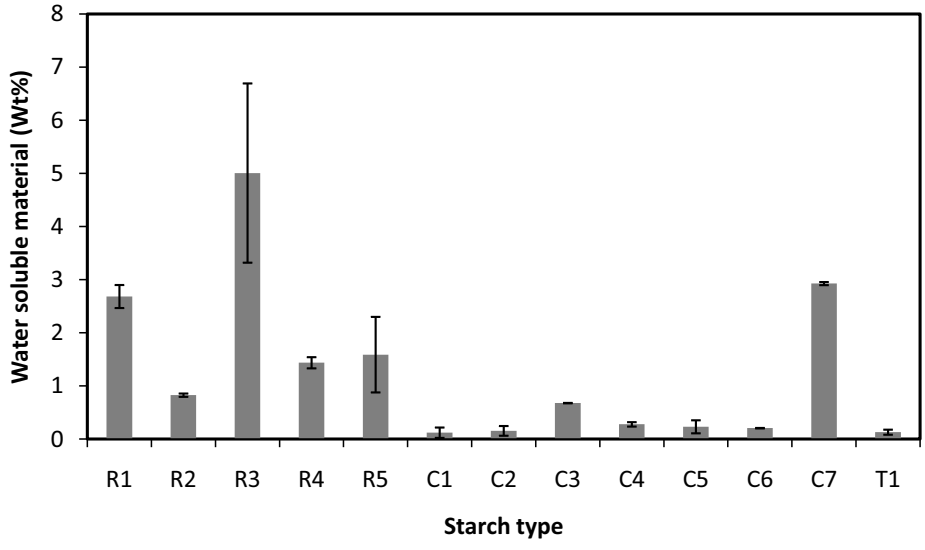

Figure 4.5: Water-soluble fraction of starch types. Error bars indicate 5 % confidence interval. R= rice starches, C= corn starches, T= tapioca starch.

Water immersion

The effect of water immersion on model paints is seen in Figure 4.6. It is evident that the corn starch C1 has the lowest weight gain in water; in fact the weight gain is close to that of the pure binder system. However, it is also seen that the coating still gains significantly more weight than the commercial reference does. This is due to the starch, which has a hydrophilic surface. The water

uptake of antifouling coatings needs to be limited, otherwise swelling will compromise the mechanical integrity of the coating, and ultimately blisters can occur.

Due to its low amount of water-soluble contaminants, and the lowest water uptake of all the starch-types tested, the corn starch C1 (C*gel 03401 from Cargill) was selected as the most suitable starch type. All the following results were obtained using this starch type.

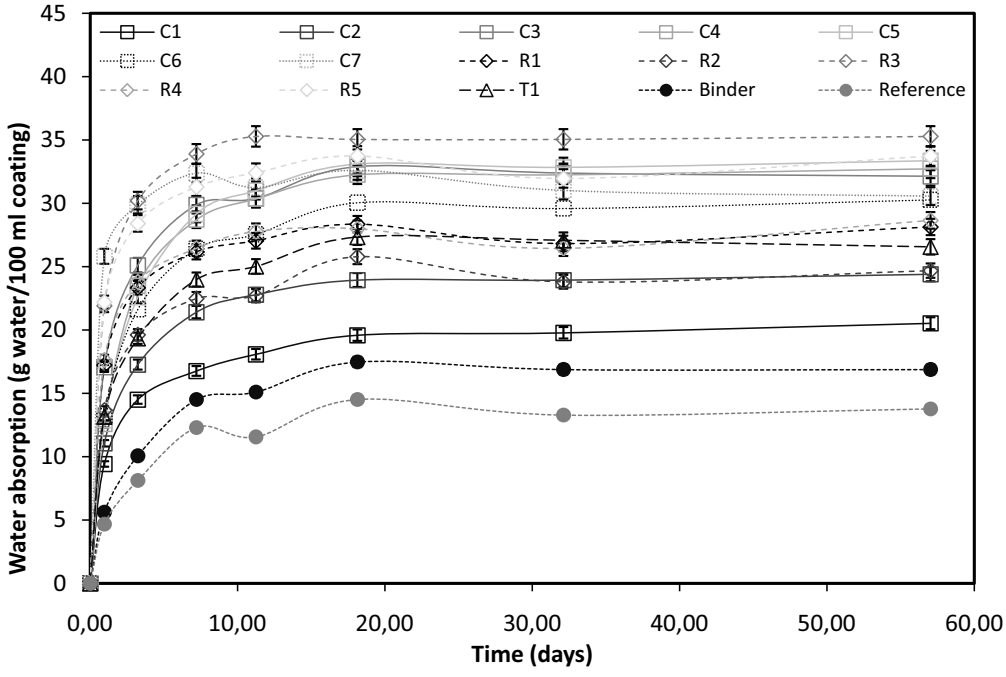

Figure 4.6: Development of weight of model coatings formulated with 30 vol% of the different starch-types. The starch types are the only difference between the coatings, and therefore the water absorption can be related to the starch. The coatings were immersed in tap water at 45 °C. The reference is the commercially available Mille Xtra from Hempel A/S.

Blister box

During four months exposure in the continuous blister box, none of the coatings tested developed blisters. This indicates sufficient mechanical stability to survive even prolonged exposure to seawater for all the coatings.

Polishing and leaching

The results of the development in dry film and leached layer thicknesses of the 12 coatings listed in Table 4.2 are provided in Figure 4.7 to Figure 4.10. It is evident, from Figure 4.7, that none of the coatings based on the A-type binder polishes measurably. Figure 4.8 shows that starch and glucoamylase induce polishing in the B-type binder. However, the dry film thickness increases initially due to swelling. Considering the decreasing part of the figure (BSG), a polishing rate of 10.1 ± 0.3 µm/month is derived. Figure 4.9 shows no sign of polishing in the three C-type coatings. In Figure 4.10, it is seen that all the D-type coatings swell considerably. However, after a delay, due to swelling, the coating, DSG polishes with a rate of 7.8 ± 0.3 µm/month.

It is seen that leached layers develop and reach stable values for all the coatings. Generally, the leached layers become relatively thick over time, ranging from 50 to 100 µm after 100 days on the rotary set-up. However, the leached layer thickness was identified using a marker, and the penetration of the marker, due to potential swelling and subsequent porosity of the coating films outer layer, may have influenced the results. The leached layer thicknesses measured are somewhat more than the desirable 5-20 µm (TBT-based coatings see Kiil et al. (2001)), but may not be problematic.

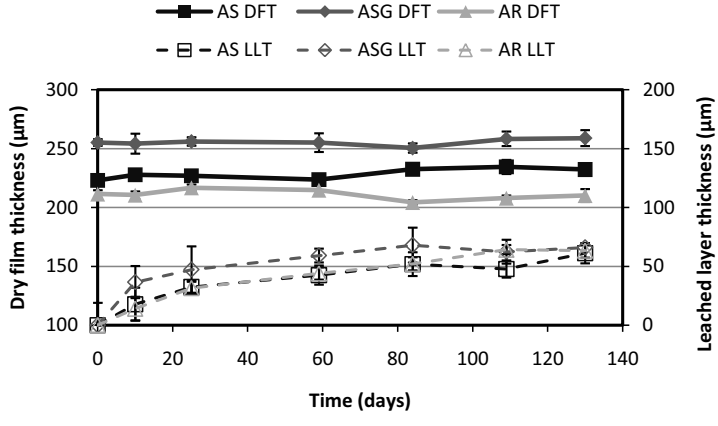

Figure 4.7: Development of dry film thicknesses (DFT) and leached layer thickness (LLT) of the coatings formulated from binder A. S=starch (no enzyme), SG=starch and glucoamylase, and R=Cu_2O and ZnO. See Table 4.2 for coating compositions. The error bars indicate the 95% confidence level of the measured thickness of the sample. The temperature was 35 ºC and the rotor was operated at 20 knots.

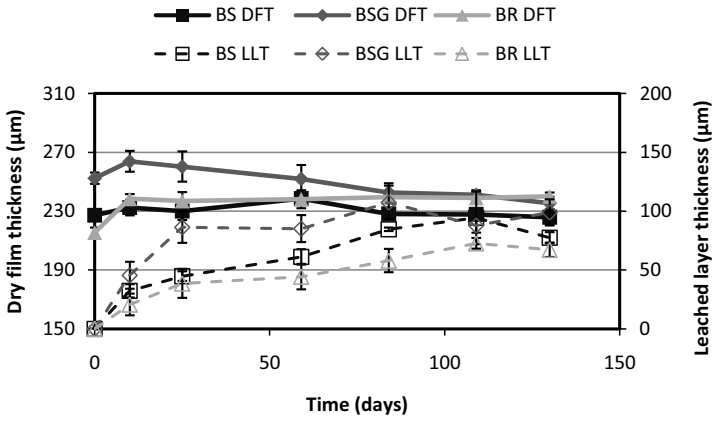

Figure 4.8: Development of dry film thicknesses (DFT) and leached layer thickness (LLT) of the coatings formulated from binder B. S=starch (no enzyme), SG=starch and glucoamylase, and R=Cu_2O and ZnO. See Table 4.2 for coating compositions. The error bars indicate the 95% confidence level of the measured thickness of the sample. The temperature was 35 ºC and the rotor was operated at 20 knots.

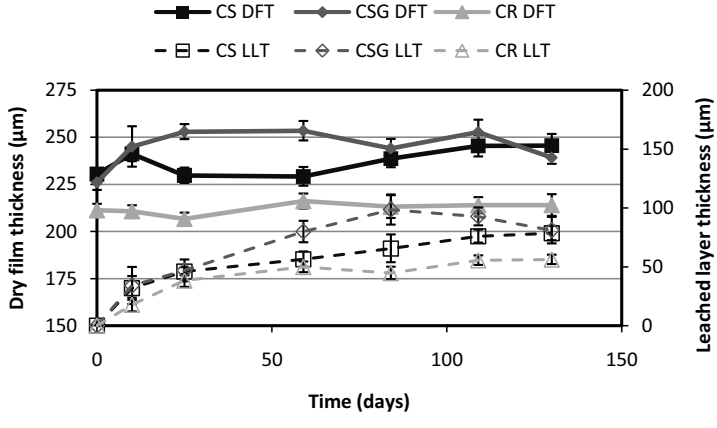

Figure 4.9: Development of dry film thicknesses (DFT) and leached layer thickness (LLT) of the coatings formulated from binder C. S=starch (no enzyme), SG=starch and glucoamylase, and R=Cu_2O and ZnO. See Table 4.2 for coating compositions. The error bars indicate the 95% confidence level of the measured thickness of the sample. The temperature was 35 ºC and the rotor was operated at 20 knots.

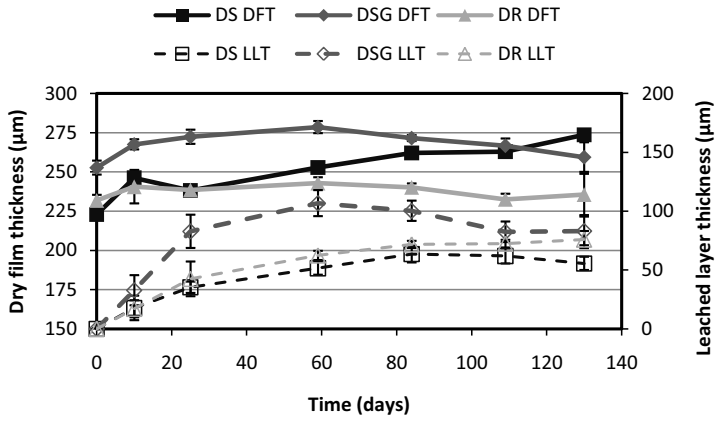

Figure 4.10: Development of dry film thicknesses (DFT) and leached layer thickness (LLT) of the coatings formulated from binder D. S=starch (no enzyme), SG=starch and glucoamylase, and R=Cu_2O and ZnO. See Table 4.2 for coating compositions. The error bars indicate the 95% confidence level of the measured thickness of the sample. The temperature was 35 ºC and the rotor was operated at 20 knots.

Discussion

The weight gain reported for the model coatings in the water immersion test can only be ascribed to the different starch-types enclosed in the binder because only the type of starch varies. Figure 4.11 shows a correlation of water taken up by model coatings against the oil absorption of the starches in the coatings. It is evident that two regimes exist. These two regimes are identified as covering starches containing either above or below 0.75 wt% water soluble contaminants. There seems to be indications that when the content of water soluble material is high enough, this material is responsible for the vast majority of water taken up by the coating. However, water uptake is not only decreased by lowering the amount of water-soluble content. Another factor is influencing the uptake of water, which may be the surface area of starch represented by the oil absorption in Figure 4.11, but this is not a strong effect. It is therefore concluded that it is a requirement, but not sufficient, that starch used as antifouling coating ingredients must contain well below 0.75 wt% of water-soluble material. Notice also in Figure 4.11 that data for rice starches appear to be more scattered than those for corn starches.

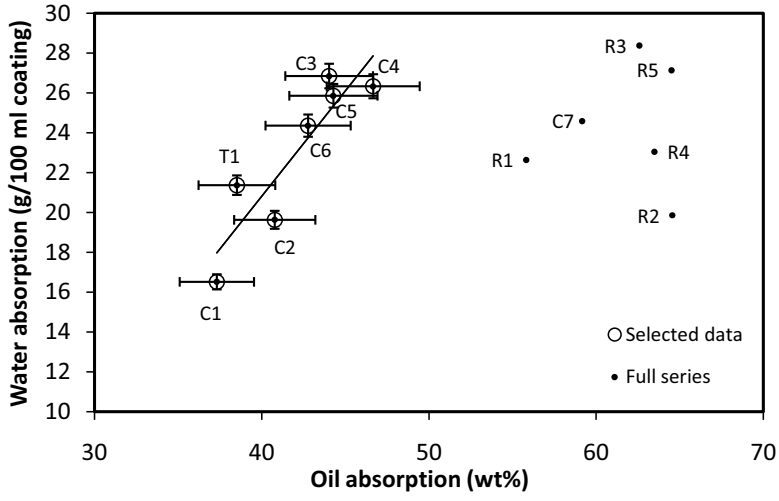

Figure 4.11: Correlation of water absorption of starch types against oil absorption. The small dots indicate all the data and the open circles show the data points for starches with water soluble content below 0.75 wt%.

From the measurements of the development of dry film thicknesses, it is evident that no polishing occurs in the coatings A and C. This corresponds to the elevated content of acrylate that retards polishing. If the acrylate is substituted by zinc resinate (coating BSG), smooth polishing is obtained. However, if the acrylate is substituted for poly(vinyl methyl ether) (coating DSG) polishing is delayed. This is due to significant swelling, which is seen when comparing the development in dry film thickness of coating CS (starch containing), with that of coating CSG (starch and glucoamylase containing). Poly(vinyl methyl ether) is more hydrophilic than the other binder constituents, therefore the coatings based on the C binder system take up more water, and swell more.

Polishing rates of 6 to 7 µm/months were found. This is suitable for antifouling coatings for commercial ships. For yacht purposes faster polishing is preferred. Faster polishing rates can be obtained by modification of the binder system. However, also the enzyme content of the starch pigment can be modified to manipulate the polishing rate. If the enzyme-activity is increased, the leached layer will develop faster, and polishing-rates will be increased, conversely lower enzyme-activity will decrease polishing rates.

Figure 4.7 to Figure 4.10 show that no polishing of cuprous oxide-based coatings occurred during the more than 20 weeks the polishing experiment lasted. This is probably due to the high amounts of retarders added to stabilise the coatings. However, it is expected that polishing of the

copper-based coatings will occur eventually, when the leached layers have been allowed to develop sufficiently.

The leached layers were identified using a marker and factors other than pigment leaching may have caused the marker to penetrate the surface of the film, such as open pores created during swelling and subsequent drying. Furthermore, the leached layer thicknesses of the coatings containing starch and enzymes are very inhomogeneous. This may be caused by the enzymes 'drilling' into the starch granules instead of pealing the granules from outside. Glucoamylase is hydrolysing starch from the reducing end of the glucopolymer and therefore the path of the enzyme is depending on the spatial distribution of the starch chains in the granule. Leached layers of the coatings containing starch and enzyme are therefore not necessarily depleted of starch in the same way as the leached layer of cuprous oxide based coatings, which is more well-defined (Kiil et al. 2001).

Starch enclosed in antifouling coatings and glucose released from the coatings may be substrates for biofouling organisms. Therefore, an unprotected coating based on polishing by starch and enzyme may be subject to biofouling. However, if a short-lived biocide efficient in preventing biofouling is released from the coating due to the polishing effect, an environmentally friendly, polishing, antifouling coating may be produced.

Conclusions

In this paper a suitable starch for use as antifouling coating ingredient has been identified. It has a very low content of water soluble material, and only little water is taken up by coatings containing this starch. The particle size distribution of the starch type is also suitable for inclusion in an antifouling coating.

Soluble matrix coatings can be formulated so that they polish due to the enzyme-mediated release of the starch pigmentation in the form of dissolved glucose. In fact, binder compositions that did not polish based on a high content of cuprous oxide, polished when the cuprous oxide was substituted for starch and glucoamylase. Polishing rates in the region of 7 to 10 µm/month was found, and whereas the polishing rates achieved here are suitable for commercial ships, they should be increased to meet yacht purposes.

Enzyme activity in the coating may be altered to change the polishing rate of the coating, and the coating composition should be optimised to minimise the adverse effects of the starch. Furthermore, the starch and enzyme pigmentation should be tested in other binder systems.

References

Dunn-Coleman, N, Neefe-Kruithof, P, Pilgrim, C E, Ward, D E, van Solingen, P. 2006.*trichoderma Reesei* glucoamylase and homlogs thereof. International application published under the patent cooperation treaty.

Kiil, S, Dam-Johansen, K, Weinell, K, Pedersen, M, S. 2001. Analysis of self-polishing antifouling paints using rotary experiments and mathematical modelling. Ind. Eng. Chem. **40**. p 3906 - 3920.

Kiil, S, Dam-Johansen, K, Weinell, C, E, Pedersen, M, S,. 2002a. Seawater-soluble pigments and their potential use in self-polishing antifouling paints: simulation based screening tool. Progress in organic coatings **45**. p 423-434.

Kiil, S, Weinell, C, E, Pedersen, M, S, Dam-Johansen, K. 2002b. Mathematical modelling of self-polishing antifouling paint exposed to seawater: a parameter study. IChemE **80**. p 45 - 52.

Olsen, S, M, Pedersen, L, T, Kiil, S, Kristensen, J, B,. 2008. Developing enzyme-based antifouilng coatings part II: the coating system. Oral presentation at the 14th international congress on marine corrosion and fouling. Kobe.

Sauer J, Sigurskjold B W, Christensen U, Frandsen T P, Mirgorodskaya E, Harrison M, Roepstorff P, Svensson B. 2000. Review Glucoamylase: structure/function relationships, and protein engineering. Biochimica et Biophysica Acta **1543**. p 275-293.

Somasekharan, K, N, Ubrammanian, R, V,.1980. Structure, mechanism and reactivity of organotin carboxylate polymers. Acs symp ser. **121**. p 165-181.

Yebra, D M, Kiil, S, Dam-Johansen, K, Weinell, C. 2005. Reaction rate estimation of controlled-release antifouling paint binders: Rosin based systems. Progress in organic coatings **53**. p 256-275.

Yebra, D M, Kiil, S, Weinell, C E, Dam-Johansen K. 2006a. Mathematical modeling of tin-free chemically active antifouling paint behaviour. AlChE **52** (5). p 1926-1940.

Yebra, D M, Kiil, S, Weinell, C E, Dam-Johansen, K,. 2006b. Dissolution rate measurements of sea water soluble pigments for antifouling paints: ZnO. Progress in organic coatings **56**. p 327-337.

5. Chapter five - Antifouling effect of hydrogen peroxide release from enzymatic marine coatings

This chapter concerns the testing of enzyme-mediated hydrogen peroxide release from marine coatings. Testing of similar coatings under temperate conditions are reported by Kristensen et al. (2009b), in their study, the antifouling effect of the coatings were exceeding that of commercial references during more than two months immersion in the North Sea. In this chapter, the same coatings are characterised in terms of common antifouling coating properties and the antifouling efficiency under more severe fouling conditions, in equatorial and Mediteranean climates are tested.

The work described in this chapter is carried out in collaboration with Jakob Broberg Kristensen and Brian Søgaard Laursen from Danisco A/S, and they are thanked for good cooperation and interesting discussions. In this chapter there is referred to two yet unpublished papers by Kristensen et al. (2009a and 2009b). The content of this chapter is intended for publishing in Journal of coating technology and research during 2009, the title of the paper will be 'Antifouling effect of hydrogen peroxide release from enzymatic marine coatings: exposure testing under equatorial and Mediterranean conditions'. (Authors: Olsen S M, Pedersen L T, Kristensen J B, Laursen B S, Dam-Johansen K, Kiil S).

Introduction

Currently the most commonly used biocide in antifouling coatings is cuprous oxide (Cu_2O). Copper in seawater and sediments are suspected of adversely affecting the marine organisms (de Oliverira-Filho et al. 2004). A potentially very attractive alternative to the present metal-based biocides is short-lived antifouling agents decomposing or reacting to harmless products. One example of such a compound is hydrogen peroxide (H_2O_2).

After release into seawater, hydrogen peroxide rapidly degrades into water and oxygen:

$$2H_2O_2(aq) \rightarrow H_2O(l) + O_2\,(aq) \qquad (5.1)$$

The decomposition reaction of hydrogen peroxide is catalysed homogeneously and heterogeneously, and evidence points towards a free radical intermediate, implying that a chain reaction is involved (Eul et al. 2001). The rapid degradation of hydrogen peroxide into water and oxygen is the reason for the very attractive properties of the compound as an active antifouling

coating ingredient because the antifouling effect, following release, is needed for a very short time only.

Previously, the antifouling effect of hydrogen peroxide in bulk solution laboratory assays was investigated. According to Zhang et al. (2008), settlement and metamorphosis of barnacles are unaffected by hydrogen peroxide concentrations up to 13.6 mg/l. In fact, settlement increases somewhat for concentrations between 13.6 mg/l (0.4 mM) and 27.2 mg/l (0.8 mM), but decreases when the hydrogen peroxide concentration exceeds 34 mg/l (1 mM) (Zhang et al. 2008) (the mechanism behind this observation was not determined by the authors). Similar results using electrochemical generation of reactive oxygen species are reported (Perez et al. 2008). In a laboratory antifouling assay, a concentration of 13.6 mg/l (0.4 mM) hydrogen peroxide inhibits barnacle settlement efficiently. However, pulsed electric fields (PEF) based on high-strength electric fields with low voltages (see Perez-Roa et al. (2008) for elaboration) inhibit biofouling more potently at lower hydrogen peroxide concentrations (1.7 mg/l), and therefore, hydrogen peroxide seems to be a precursor for the actual antifouling agent, which supposedly is the hydroxyl radical (Perez et al. 2008). This theory is supported by the investigations of Elzanowska et al. (1995), hydrogen peroxide alone is not very toxic to prokaryotic cells, but addition of low-valent transition metal ions like ferrous or ferric complexes or water-soluble salts of cuprous or cupric ions (note that Cu_2O is a common antifouling biocide) improve the hydrogen peroxide oxidative degradation of various biological compounds, due to the generation of hydroxyl radicals (HO·) (Elzanowska et al. 1995). Hydrogen peroxide together with ferric ions, inhibit mussels (*Mytilus galloprovincialis*), polychaetes (*Hydroides norvegicus, Dexiospera foraminosa*), brozoa (*Bugula dentate*), hydroid *(Obelia*) and colonial tunicates from settling (Nishimura et al. 1988). Barnacle larvae (*Balanus amphitrite*) are not affected during settling; however, after metamorphosis, the barnacle growth-rate is reduced by about 50 % (Nishimura et al. 1988). Also, a relatively low concentration (0.07 mg/l) of hydrogen peroxide is reported to inhibit mussel biofouling significantly, and addition of iron sulphate improves the effect (Nishimura et al. 1988). This effect is also reported in another study where the combination of hydrogen peroxide and iron sulphate in bulk solution inhibits biofouling to 1/6 of the control (Ikuta et al. 1988). In a coating based experiment, precursor inorganic peroxides have been used in hydrogen peroxide based antifouling (Olsen et al. 2008). The zinc peroxide based antifouling coating performs better than a similar coating based only on zinc oxide (same pigment volume concentration), and this effect is achieved by a hydrogen peroxide release rate of 7.48 µg/(cm^2·day) equivalent to 0.22 µmoles/(cm^2·day)

(Olsen et al. 2008). Furthermore, photo catalytic hydrogen peroxide generation in-situ in antifouling coatings using anatase coated zinc oxide is described in (Morris and Walsh 1996). However, no estimation of the antifouling effect is available in the patent. For a more elaborate discussion on the fate and effect of hydrogen peroxide as antifouling agent see Appendix I.

Enzyme-based antifouling has been known and studied over the past 20 years. For reviews of approaches to and mechanisms of enzymatic antifouling see (Kristensen et al. 2008) and (Olsen et al. 2007(see chapter two)). Enzyme-mediated controlled release of hydrogen peroxide has been described in Hamade et al. (1998) and (Poulsen and Kragh 1999). The approach of (Poulsen and Kragh 1999) exploits the fact that hydrogen peroxide can be produced during oxidation of glucose by the enzyme hexose oxidase (HOx):

$$C_6H_{12}O_6(aq) + O_2(aq) \xrightarrow{HOx} C_6H_{10}O_6(aq) + H_2O_2(aq) \quad (5.2)$$

Sucrose is too water-soluble to be used directly as antifouling coating ingredient (Kiil et al. 2002), and as glucose is even more water-soluble, it needs to be produced in-situ. This can be done by the enzyme glucoamylase (GA), which 'cuts' glucose units from starch:

$$(C_6H_{10}O_5)_n(s) + H_2O(l) \xrightarrow{GA} C_6H_{12}O_6\ (aq) + (C_6H_{10}O_5)_{n-1}(s) \quad (5.3)$$

Equation (5.3) is hydrolytic, and no starch conversion will therefore occur in the absence of water. In a solvent-based coating system containing starch, glucoamylase and hexose oxidase, the production of hydrogen peroxide will first be initiated when the applied, dried coating is immersed in water. If the rate of glucose productionin (5.3) is lower than the rate of consumption in (5.2), the reaction rate of (5.3) becomes the rate-controlling step for hydrogen peroxide release.

A simplified illustration of the overall mechanism of hydrogen peroxide release from an immersed antifouling coating containing starch and the enzymes glucoamylase and hexose oxidase is shown in Figure 5.1. Glucoamylase (GA) is an exo-amylase meaning that it hydrolyses glucose units of off starch. This happens in the wetted outermost layer of the coating. Glucose is then oxidised to gluconolactone by dissolved oxygen that is reduced to hydrogen peroxide. The reaction is catalysed by hexose oxidase.

Enzymes are attractive potential antifouling coating ingredients because they are easily degradable proteins, and can therefore, presumably, be added to antifouling coatings without harmful environmental consequences (see elaborate discussion on this point in (Kristensen et al. 2008). Antifouling achieved with hydrogen peroxide produced by enzymes and starch, and ultimately resulting in water and oxygen (and gluconolactone) is therefore considered to be environmentally benign.

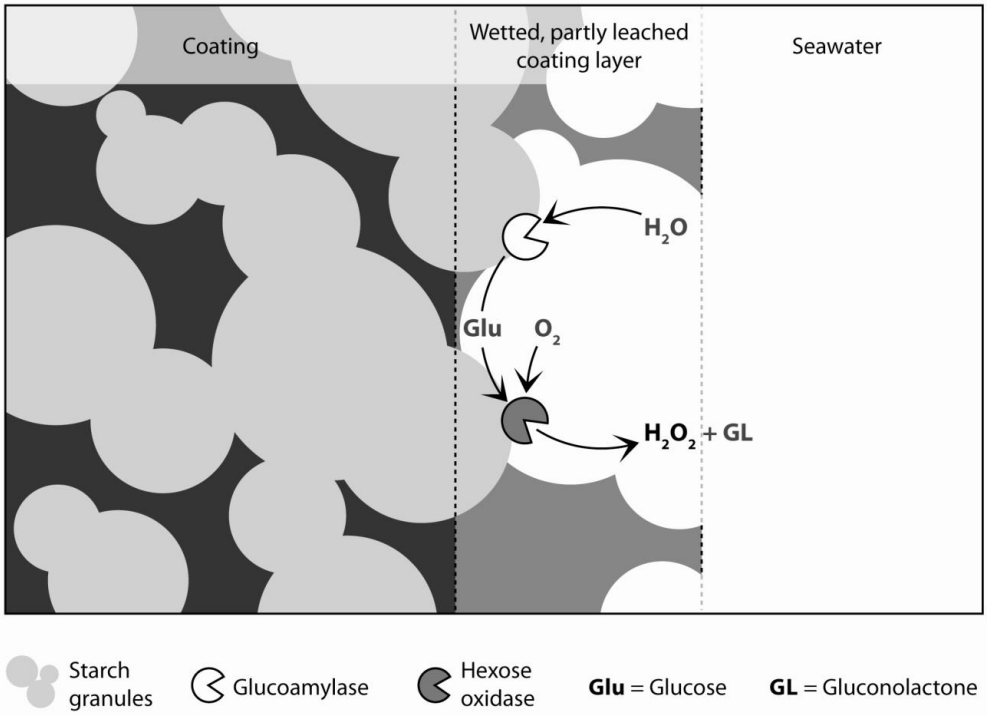

Figure 5.1: Schematic illustration (cross-section view) of the mechanisms of enzyme-based hydrogen peroxide release from an antifouling coating. Glu= glucose, GL= gluconolactone, the white and grey enzymes in the figure is glucoamylase attached to starch and silica-enclosed hexose oxidase respectively. As opposed to normal antifouling coatings, the leached layer is not as well defined in the starch and glucoamylase-containing coatings.

Strategy of investigation

The focus of this paper is to evaluate the antifouling potential of enzyme-mediated controlled release of hydrogen peroxide under more severe biofouling conditions: In seawater outside Singapore, where the climate is equatorial, and in the Mediterranean outside Barcelona, where the climate is dry summer subtropical (Mediterranean climatic conditions). A novel coating ingredient composed of starch and enzymes has been prepared by spray drying, and coatings have been formulated containing the new ingredient. Common coating characteristics have been evaluated, and transient hydrogen peroxide release rates from the coatings, using artificial seawater, have been monitored under laboratory conditions. An approximate target of hydrogen peroxide release rate is

between 1 and 10 µg/(cm^2·day) based on starch availability and the required release rate reported in (Morris and Walsh 1996).

Experimental materials and methods

Starch and enzyme preparation

Starch and enzymes were prepared as described in (Kristensen et al. 2009b). Corn starch was obtained as C*gel 03401 from Cargill. According to the producer, the average particle diameter of the starch is 15 µm. Its gelatinisation temperature is between 62 and 71 ºC so that application temperatures must be kept below 62 ºC to avoid gelling of the coating.

Glucoamylase and hexose oxidase was obtained from Danisco (Glucoamylase is more detailed described in Dunn-Colemann et al. (2007) and the hexose oxidase is described in Poulsen and Kragh (1999)). Prior to spray drying, hexose oxidase was encapsulated in porous silica using polyethylene amine as described in Kristensen et al. (2009a) (d_p~5 µm). Starch, glucoamylase, and silica-encapsulated hexose oxidase were spray dried from water-based slurry. Starch (75 g) was added together with an aqueous solution of glucoamylase with concentrations ranging from 0.012 to 1.2 glucoamylase units/mL (1 glucoamaylase unit corresponds to the amount of enzymes releasing 1 g glucose production per hour) to a silica-encapsulated hexose oxidase suspension in a concentration of 16 units/g (1 unit corresponds to the amount of enzymes consuming 1 micromole of glucose per minut). Air inlet temperature was 135 °C, and the air and powder outlet temperature was 80 °C. At the spray nozzles, water cooling with 0 °C water was used. The apparatus used was a Mini Spray Dryer B-191 from Buchi laboratory equipment. The result of the spray drying is a heterogeneous powder. One component of the powder is starch-glucoamylase particles (d_p~15 µm see Figure 5.3) where glucoamylase is attached to starch via a starch binding domain (Sauer et al. 2000) (hydrogen and van der Waals bonds). The other type of particles in the powder is hexose oxidase enclosed in porous silica (dp~5 µm (Kristensen et al. 2009a)). Due to differences in particle size (15 and 5 um, respectively), some segregation of the two types of particles in the spray dry powder product may occur. This could potentially lead to powder sampling problems during coating formulation. However, for the coatings considered here, segregation was not a cause of limited hexose oxidase activity as can be seen in Figure 5.12 and the discussion associated with this figure. It was not investigated if the smaller silica particles bind to the larger starch particles during spray drying because such a particle interaction is expected to be broken down during pigment dispersion.

If a coating is successfully formulated, then separation of the enzyme-particles would ideally result in a coating that produces glucose in the deepest part of the leached layer, and while diffusing through the leached layer glucose will pass hexose oxidase, and hydrogen peroxide will be produced. This is important because separation of hydrogen peroxide from the enzymes ensures that hydrogen peroxide does not affect enzyme stability.

Critical pigment volume concentration

The critical pigment volume concentration (CPVC) of the starch-pigment was estimated from the oil absorption obtained using commercial grade linseed oil (Sauer et al. 2000).

Water-soluble content of starch ingredient

The water-soluble content of starch-based ingredients was determined gravimetrically. Starch material was mixed in deionised water at room temperature and stirred effectively. The slurry was then centrifuged at 15,000 rpm for ten minutes, and the dry matter content of the supernatant was determined by water evaporation and weighing.

Formulation of coatings

Some of the starch properties could potentially compromise the mechanical stability of the coatings (see the Results section). Therefore, a high amount of acrylic retarder was added to counteract the effect of the starches. This meant that the binder composition was of the insoluble matrix type. All the coatings had a starch content of 30 vol%, and the remaining 70 vol% of the coating consisted of 36 vol% zinc resinate (produced from rosin Cas no 65997-06-0 and commercial grade zinc oxide), 18 vol% methyl methacrylate/n-butyl methacrylate/methacrylic acid terpolymer (100/100/1 molar ratio. Purchased as Degalan LP 64/12 from Rohm GMBH) and 6 vol% poly(vinyl methyl ether) (Purchased as Lutonal M40, 45 % from BASF AG). Inert red iron oxide pigment (Cas nr: 1309-37-1) was added (10 vol%) to obtain suitable mechanical properties of the coatings (Yebra et al. 2006a). Eight coatings with a composition of the experimental pigmentation as provided in Table 5.1 were prepared.

The coatings were produced on two-speed Diaf 37-33v mixer at low speed, using a dissolver turbine disk of 5 cm in diameter, and 250 ml cans with a diameter of 6.5 cm. Dispersion was complete when the liquid paint reached a fineness of grind below 60 µm.

Table 5.1: Coatings formulated to test enzyme-mediated hydrogen peroxide release from antifouling coatings. Glucoamylase levels are provided in arbitrary units (AU) relative to coating A. These should be indicative of the level of hydrogen peroxide release. Presence or absence of hexose oxidase is indicated by (+) and (-) respectively.

	Starch (vol%)	Glucoamylase (AU)	Hexose oxidase	Cu_2O (vol%)	ZnO (vol%)	Compounds released from the coating
Coating A	30	1	+			H_2O_2 α-D-gluconolactone
Coating B	30	10	+			H_2O_2 α -D-gluconolactone
Coating C	30	50	+			H_2O_2 α -D-gluconolactone
Coating D	30	100	+			H_2O_2 α -D-gluconolactone
Coating G	30	0	+			
Coating H	30	50	-			Glucose (level 50)
Coating I	30	0	-			
Coating L	0	0	-	22.5	7.25	$CuCl_2^-$; $CuCl_3^-$; Cu^{2+} (Kiil et al. 2001) Zn^{2+}; $ZnCl_4^{2-}$; $Zn(OH)_4^{2-}$ (Yebra et al. 2006b)

Particle size distribution

The particle size distributions of pure starch powder and the powder after spray drying (containing both starch-glucoamylase particles and silica enclosed hexose oxidase particles) were measured using a Malvern Mastersizer 2000 from Malvern Instruments, and a Hydro 2000G sample disperser. Measurements on the pure starch compound were done on starch suspended in ethanol. Measurements of the particle size distribution of the starch-pigment, after it had become part of a coating, were performed by taking a few drops of the liquid paint and dispersing in commercial grade xylene and measure directly on the slurry.

Water immersion test

Coatings were applied on 5·10 cm^2 polycarbonate panels and the panels immersed in tap water adjusted to 45 °C. The purpose of this was to monitor the water uptake, and potential failures of the coatings due to water immersion. Water of low salinity and increased temperature was used to

accelerate the mechanism leading to failure of the coatings. The panels were monitored gravimetrically during five weeks immersion. Before weighing, excess water was gently wiped off the panels. A commercially available antifouling coating (Mille light from Hempel A/S) was included in the test series as reference.

Blister box

The coatings containing starch and glucoamylase were tested for their tendency to blister (a typical coating defect) according to ASTM D 4585. The coated panel surfaces were exposed to 40 °C saturated water vapour, at an angle of 60° to the horizontal, and the back of the panels exposed to room temperature. The coatings were inspected every second week for three months, and potential blistering was evaluated according to ASTM D714.

Antifouling efficiency of hydrogen peroxide releasing coatings

Antifouling efficiency of enzyme-mediated hydrogen peroxide release from the formulated antifouling coatings (see Table 5.1) was tested by static immersion of coated panels in seawater (see Kiil et al. 2006). Polycarbonate fibre panels with an area of 10·20 cm^2 and a thickness of 0.5 cm were used. The panels were first primed using Hempatex high-build 4633 from Hempel A/S (a system based on chlorinated rubber binders). This was done to improve adhesion strength between panel and experimental coating. The experimental coatings were applied using 8 cm Dr Blade applicator with a gap of 350 µm. The (uncoated) edges of the panels were covered in commercial antifouling coating to prevent biofouling from developing from the unprotected edges of the panels (this was inadequately done on panel coated with coating L for immersion in Singapore, which will be elaborated in the Results section). Panel exposure was done statically on a raft in Singapore (1^o 23'33 N, 103^o 58' 34 E) for eight weeks during June and July 2008. The climatic conditions at the site are equatorial, and the coatings were therefore subject to maximal biofouling stress (Kiil et al. 2006). The water depth at the raft site was 3 meters, the salinity 3.2 to 3.5 wt% and seawater temperature varied between 32 °C and 35 °C. The panels were allowed to foul for 8 weeks before inspection (minimum testing time at this site to ensure sufficiently biofouling on a blank panel).

Panels were also mounted on a raft in the Mediterranean outside Barcelona, where the biofouling intensity is lower than in Singapore, but still quite severe and somewhat diversified from a biological point of view. At this site, the temperature varied between 19 and 27 ºC with an

average of 24 ºC during the exposure period, which ranged from week 22 (end of May) 2008 until week 36 (start of September) 2008.

At both sites, two commercially available antifouling coatings were included as references. These were Mille Light and Mille Xtra from Hempel A/S. Mille Light is recommended for use where there are many environmental requirements, and Mille Xtra is a copper-containing commonly applied yacht antifouling coating. Furthermore, uncoated acrylic panels were also used as references.

After the seawater immersion in Singapore had ended, the biofouling organisms were removed from the coated panels and the coatings were examined. A blue marker was used to identify the leached (porous) layer of the coatings and cross sections of coating fragments were inspected using light microscopy (Kiil et al. 2001). Also, to see if there was enzyme activity left in the coatings, fragments of the coatings from Singapore, were mixed with peroxidase and ABTS (as described below).

Release rate of hydrogen peroxide from experimental coatings

Preliminary experiments (unpublished) showed that the starch-pigment in coating C kept the highest release rate of hydrogen peroxide over time, when enclosed in a model coating. Therefore, coating C was used when monitoring the hydrogen peroxide release rate over time in a laboratory assay. The increase of hydrogen peroxide concentration in a constant volume of artificial seawater was used as a measure of the release rate. Hydrogen peroxide concentrations were determined spectrophotometrically using a coupled enzyme assay based on 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). ABTS reacts with hydrogen peroxide in a peroxidase-catalysed reaction, and hydrogen peroxide concentrations can be determined indirectly as absorbance by oxidised ABTS at 405 nm. The assay has been modified from that described by Savary et al. (2001) (used in our work to measure enzyme activity). Coating samples were applied in a six well micro-titter plate, and the plates were kept in artificial seawater at stable temperatures ranging from 4 to 35 º C. Measurements of the release rates from the coatings were performed at room temperature to eliminate the temperature effect on the reaction rates. The flux (i.e. 'J' in Table 5.2) was calculated as the slope of the development in hydrogen peroxide concentration during 60 minutes.

The stability of hydrogen peroxide release was monitored by keeping the samples in a holding tank (volume of approximately 10 L) containing artificial seawater (Grasshof et al. 1999) at

controlled temperatures. The release rates were measured after 1, 3, 7, 14 and 21 days. For measurements, the micro titter plate was removed from the container, briefly rinsed, and fresh artificial seawater was applied.

To see if glucose supply (reaction (5.3)) or oxidation (reaction (5.2)) was limiting hydrogen peroxide release (i.e. compare stability of the first, glucose releasing enzyme with the second, hydrogen peroxide producing), measurements were also performed with glucose (50.6 mM) added to the reaction solution.

The leaching properties of the coatings was monitored using a rotary system similar to the one described in Kiil et al. (2001) (polishing was not expected due to the choice of binder composition). The temperature was kept between 32 and 35 ºC, and the pH of the artificial seawater was frequently adjusted to 8.2 using 1 M hydrochloric acid or sodium hydroxide. The rotor was operated at 20 knots during the experiment. Samples were prepared as described by Olsen et al. (2008).

Results

With the overall aim to test the antifouling effect of the coatings reported by Kristensen et al. (2009b) under more severe biofouling conditions, the results listed below have been obtained. Initially, general coating properties are described. This is followed by a listing of the results obtained from the static immersion under equatorial and Mediterranean climatic conditions, and finally the hydrogen peroxide release rates measured over time is described.

Critical pigment volume concentration

The oil absorption of the starch was measured to be 37 ± 2 g oil/100g pigment. This corresponds to a critical pigment volume concentration of 63 ± 1 vol%. In addition to starch, the coatings also contained iron oxide pigments, with a critical PVC value of 58 vol%. The critical pigment volume concentration of a coating containing 10 vol% of iron oxide pigment and 30 vol% starch-pigment have been calculated to be 70 vol% and thus the reduced PVC, λ (λ=PVC/CPVC) for the coatings described in this paper is 57 vol%. This means that the coatings formulated are far from the critical PVC, and considerably more starch can be added without reaching the critical PVC.

Water-soluble content of starch pigmentation

The water-soluble content of the starch pigmentation measured is shown in Figure 5.2. It is seen that the pure starch (I) had the lowest content of water-soluble material. Total amounts of water-soluble material in the starch were greatly increased by adding glucoamylase and modified hexose oxidase. This is probably caused by the degradation of starch which is initiated when starch and enzymes are mixed in a water-based slurry. The amount of water-soluble material is high for practical application, and therefore a coating composition containing high amounts of hydrophobic retarder (co-binder) was selected. However, this approach reduces the polishing rate of the coating significantly.

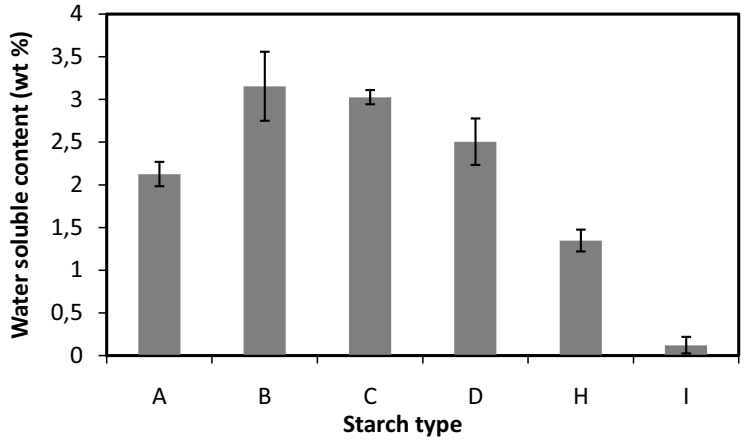

Figure 5.2: Water-soluble content of starch pigments (spray dried starch and enzymes). The letters refer to the coatings in which the pigments were applied (Table 5.1). Error bars indicate the 95% confidence levels.

Particle size distribution

The results of the particle size distribution measurements are seen in Figure 5.3. It is seen that the starch size does not alter during coating formulation (incl. dispersion step). Furthermore, as the starch supplier reports the mean particle size to be 15 µm, it seems that there is no tendency towards agglomeration. In (Kristensen et al. 2009a) the particle size of silica-enclosed hexose oxidase is reported to be distributed around 5 µm, and in Figure 5.12 no particles are seen in that region. Therefore, the silica-enclosed hexose oxidase seems to have been broken down to smaller particles during dispersion. However, only a very small fraction of the starch powder is silica-enclosed hexose oxidase, which may be the reason why it is difficult to identify in Figure 5.12.

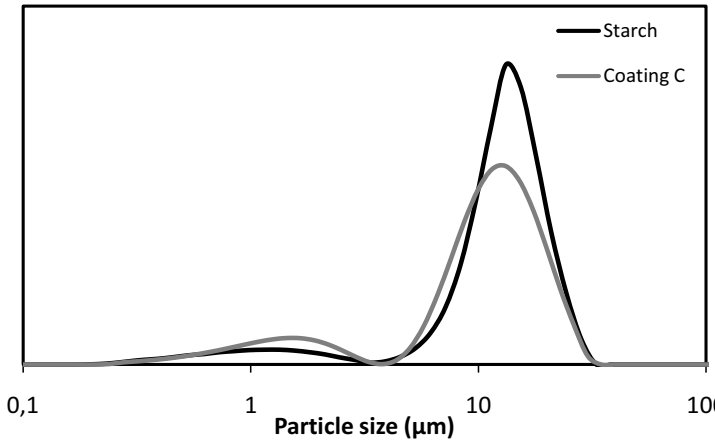

Figure 5.3: Particle size distribution (equivalent volumetric spherical diameter (Kiil et al. 2007)) of starch powder and particles in coating C. Coating C contains inert iron oxide in addition to the starch-enzyme ingredient. The iron oxide pigmentation is distributed around 2 µm in diameter according to the supplier, and the silica-enclosed HOx have a mean particle diameter of approximately 5 µm (not seen on the figure due to very low content).

Water immersion test

The transient development in the weight of the coated panels, immersed in water for 7 weeks, is shown in Figure 5.4. Coating D developed blisters rapidly after immersion, and therefore the coating was excluded from the figure. The many blisters developed in coating D is probably due to the rapid production of water-soluble glucose in this coating.

It is seen in Figure 5.4 that all the coatings containing starch and glucamylase had almost the same weight gaining profile. The steady-state weight gain is around 40 g/100 ml for these coatings, this is relatively high for practical applications, but it should be noticed that the weight gain of the commercially available antifouling coating (Ref) is approximately 30 g/100 ml coating. Therefore, it is believed that the coatings mechanical properties suit the requirement for an antifouling coating.

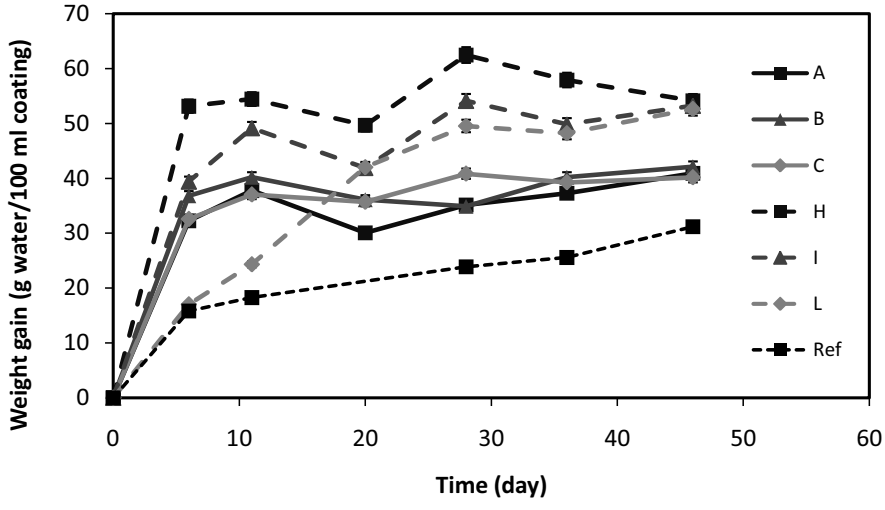

Figure 5.4: Weight gain in g/100 ml coating for the coatings included in the water immersion test. Coating thickness was approximately 200 µm in all cases and the temperature was 45 ºC. Coating D is excluded from the results due to heavy blistering. Error bars indicate the 95% confidence interval on an individual measurement.

Blister box

During four months in the blister box, no blisters had developed in any of the coatings. Pictures of the coatings A, B, C, D and L after two months in the continuous blister box can be seen in Figure 5.5. This degree of blister resistance is usually sufficient (experience of Hempel A/S) to guarantee stability of antifouling coatings in seawater. The fact that blisters developed in coating D in the water immersion test, but not in the blister box is probably due to the increased temperature (45 ºC) of the water immersion test, the blister box assay is performed at 40 ºC, however the panels are not submersed in the blister box.

An unintended consequence of the blister box assay was the occurrence of mould on starch-based coating films. Figure 5.5 shows the picture of the panels A, B, C, D, and L (from left to right) after four weeks in the blister box. It is also clear that the mould growth is inversely proportional to the enzyme activity and thus the higher hydrogen peroxide release, the lesser the mould growth. However, there is no sign of mould on the copper-containing reference (panel L), and there are no previous records of mould growing on the panels are available. This indicates that the hydrogen peroxide does inhibit microbial growth when released in sufficient quantities. However, the

coatings themselves enhance growth of the micro organisms. Coating factors potentially contributing to the growth of mould can be the starch content of the coating and/or the release of gluconolactone, as a by-product of the second enzyme reaction. Gluconolactone resembles glucose and may be a substrate for the micro organisms.

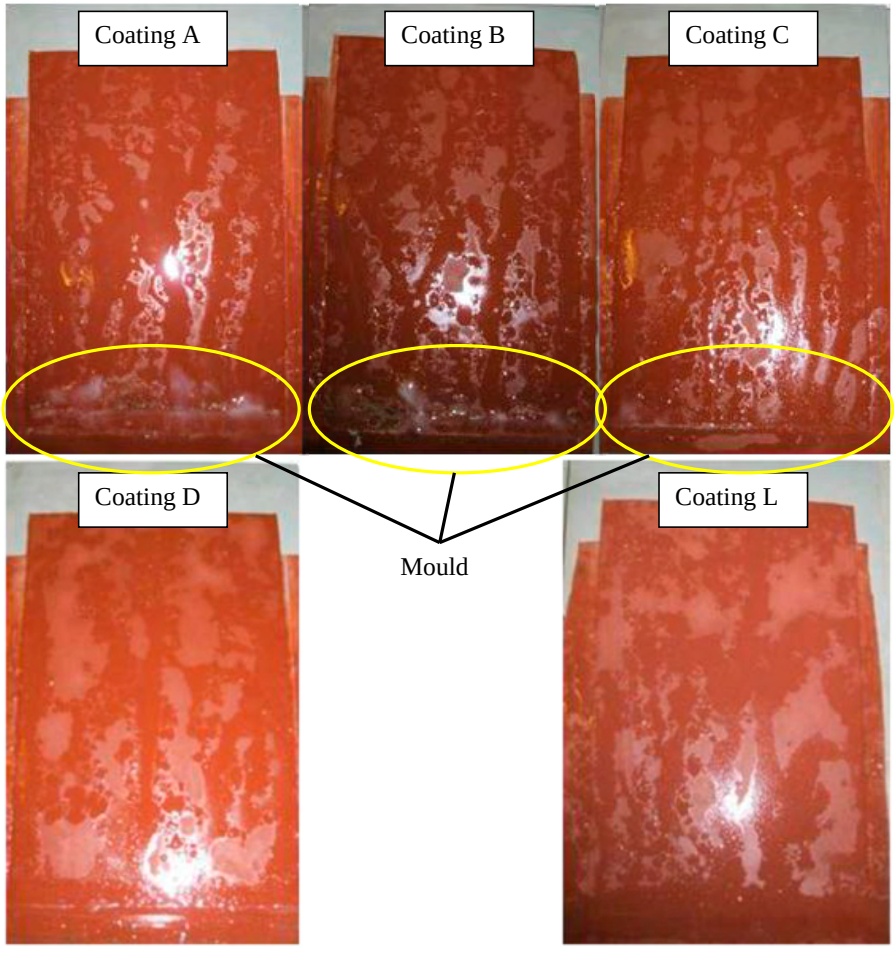

Figure 5.5: Pictures of coated blister box-panels after 28 days in a continuous blister box. Mould is identified at the bottom of the panels. The bright white spot on all panels is due to flash reflection. From left to right the panels are: A, B, C, D, L. See Table 5.1 for coating compositions.

Antifouling efficiency of hydrogen peroxide releasing coatings

The coated panels immersed in Singapore were inspected after eight weeks, and pictures were taken to document the antifouling performance of the coatings. Figure 5.6 shows the biofouling accumulated on the panels during eight weeks immersion in The Indian Ocean outside Singapore. The edges of the cuprous oxide containing reference (coating L) were not sufficiently broad to fully cover the (otherwise unprotected) edges of the panel, therefore some biofouling developed in the very small area between the experimental coating and the coating applied on the edges. This should be disregarded when interpreting the results. The biofouling that had accumulated on the experimental coatings was primarily composed of barnacles and tubeworms. However, slime and algae are identified on the two commercial references, and the diversity of biofouling organisms on the acrylic blank is greater than that on the coated panels.

Figure 5.6: Photos of coated panels after 8 weeks immersion in The Indian Ocean. ML (Mille light) and MX (Mille Xtra) are commercial references. The seawater temperature was 32-35 °C. Details of the various coatings are provided in Table 5.1.

The cross-sections of the coatings that were inspected after immersion time ended, using light microscopy, revealed that the coatings were not completely leached of starch. However, it was not practically possible to measure the leaching depth.

The fragments of the coatings that were used to test for remaining hydrogen peroxide-releasing activity showed a green colouration for the coatings A, B, C, D. This indicates that the coatings were still able to produce hydrogen peroxide. The test was not quantitative and though it revealed that there was still enzyme activity in the coatings, it was not possible to establish the actual release rates. However, the activity may have been caused by enzymes that had been dormant in the coating, during immersion, due to entrapment within the deepest coating layers.

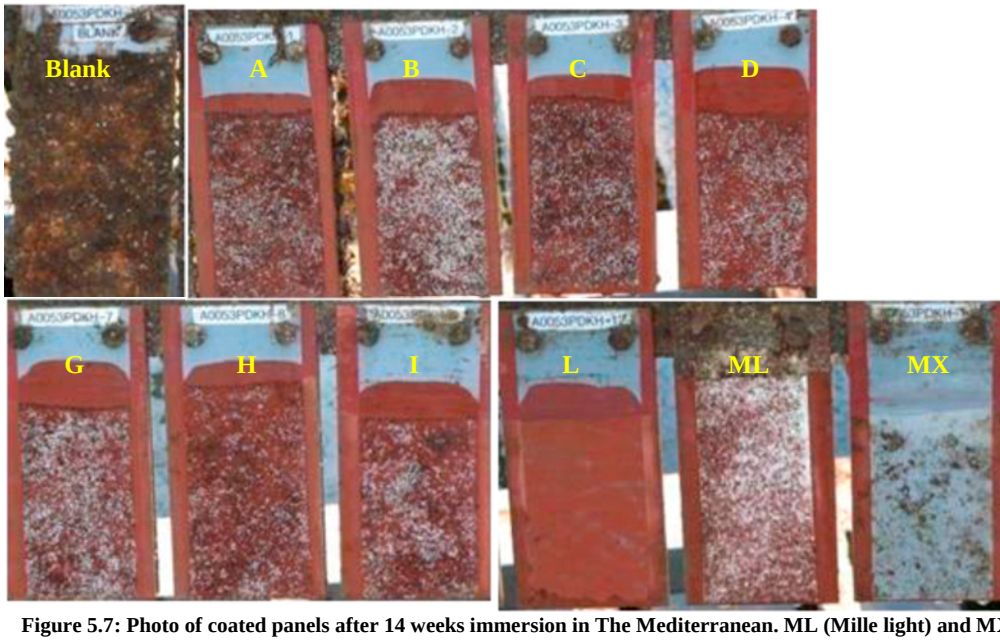

Figure 5.7: Photo of coated panels after 14 weeks immersion in The Mediterranean. ML (Mille light) and MX (Mille Xtra) are commercial references. Average seawater temperature was 24 °C. Details of the various coatings are provided in Table 5.1.

Figure 5.7 shows the pictures taken of the coatings immersed in the Mediterranean outside Barcelona after 14 weeks. It is seen that none of the enzyme-based coatings were able to stay biofouling free for this period. In fact, it is not possible to distinguish the hydrogen peroxide releasing coatings (A, B, C, and D) from the coating releasing glucose (coating H), or nothing at all (coating I). The biofouling is very diverse on the acrylic blank, but on the experimental panels the

biofouling species seems to be dominated by tubeworms, and primarily algae and slime are present on the commercial reference coatings.

The antifouling potential of coating C under temperate climatic conditions has previously been reported (Kristensen et al. 2009b). Figure 5.8 shows one of the figures used to illustrate the antifouling effect of coating C (similar to the coating C described in this paper) after 67 days immersion in the North Sea outside Den Helder, the Netherlands (Kristensen et al. 2009b). The tests were performed in duplicates, and pictures of both panels are presented. It is evident, from Figure 5.8, that coating C is the best performing of the four coatings, including the two commercial references included in the test series. Also, it is seen that the biofouling is, primarily, made up of micro-foulers.

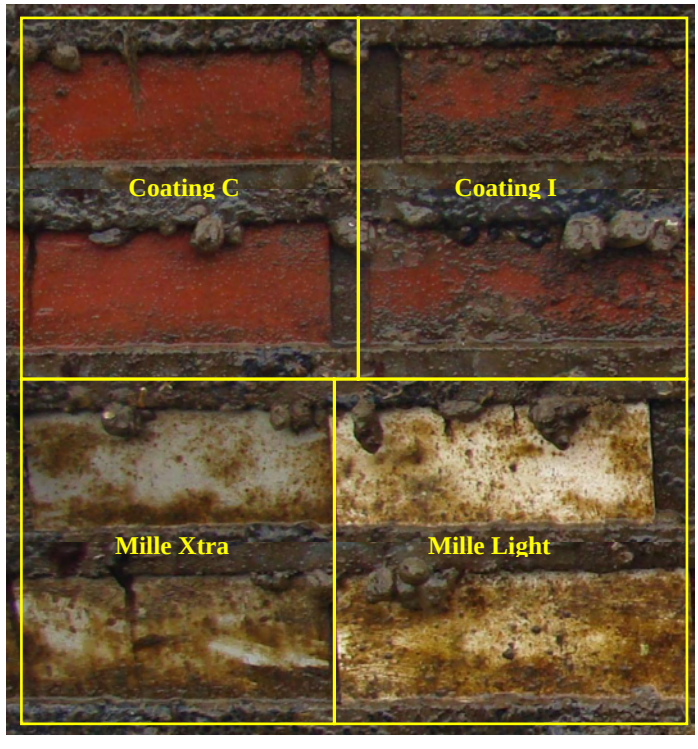

Figure 5.8: Photos of coated panels tested in the North Sea after 67 days immersion. Each coating is shown with a replicate, but they were not mounted on the raft with the same proximity. Photo modified from Kristensen et al. (2009b).

Release rate of hydrogen peroxide from experimental coatings

In Table 5.2 the hydrogen peroxide release rates for coating C after one day exposure to artificial seawater is listed and Figure 5.9 shows the stability of hydrogen peroxide release from the coating during three weeks immersion in artificial seawater at different temperatures. It is seen from the figure that the half-life of hydrogen peroxide release rate is very much dependent on temperature. This is emphasised in Figure 5.10, where a plot of half-life of hydrogen peroxide release for coating C against temperature is shown.

Table 5.2: Release rates of hydrogen peroxide from coating C after one day exposure to artificial seawater in the laboratory, at the temperatures provided in the table. Actual measurements were performed at room temperature. J is the release rate and errors are calculated as 95% confidence levels.

T ºC	$J(H_2O_2)$ µg/(cm^2·day)
4	20±1
10	24±1
15	23±2
20	27±1
25	16±2
30	30±5
35	37±3

The release rate of hydrogen peroxide from coatings C (the most hydrogen peroxide releasing coating over time) at 35 ºC from Figure 5.9 has been extrapolated to 56 days corresponding to the point of inspection in the Indian Ocean. After eight weeks, a release rate of 10^{-7} µg/(cm^2·day) is expected from coating C. This figure is much lower than the target release of 1 to 10 µg/(cm^2·day). Furthermore, the release rate decreased to below 1 µg/(cm^2·day) within the initial 11 days of immersion. Even though the extrapolation is uncertain, it is expected that the optimal conditions are found in the laboratory assays, and therefore, the real-life release-rates may well be even lower. This is probably the reason why all the hydrogen peroxide releasing coatings are insufficient in terms of antifouling effect when tested in seawater outside Singapore. The accumulated hydrogen peroxide release can be used to calculate the starch consumption over time. At 35 ºC around 5.9 vol% of the starch initially in the coating had been consumed during the 56 days the experiment lasted. This means that the decrease in hydrogen peroxide release rate is not due to depletion of starch.

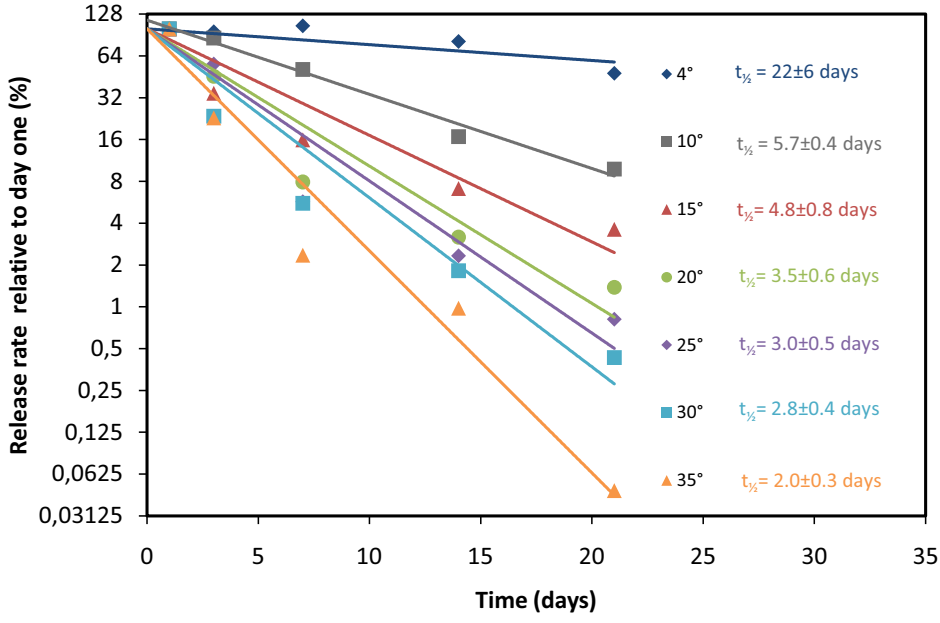

Figure 5.9: Transient development in hydrogen peroxide release from coating C as a function of temperature (see Table 5.1 for coating composition). The release rate is plotted as the absolute release rate against the release rate after day one. $t_{½}$ values have been estimated assuming a first order dependency of the flux on time. See Table 5.2 for numerical release rates measured day one.

The predicted release rate (using Figure 5.9) from the most hydrogen peroxide releasing coating after 14 weeks in the Mediterranean is 10^{-9} µg/(cm^2·day) (which is negligible for practical purposes), and the coating reached a release rate of hydrogen peroxide of 1 µg/(cm^2·day) after 13 days immersion. Therefore, the poor antifouling ability of the coatings is caused by a rapid drop in hydrogen peroxide release rate. In the Mediterranean, the theoretical starch consumption after 14 weeks did not exceed 3.7 % of the initial amount, meaning that starch depletion was not responsible for the loss of activity.

The average temperature in the North Sea was around 17 ºC during the immersion period (Kristensen et al. 2009b). Significantly improved lifetime of the coatings is therefore expected compared to the warmer climate in Singapore. Extrapolating for a temperature of 15 ºC (see Figure 5.9), the release rate of hydrogen peroxide from coating C in the North Sea is expected to approximate 10^{-2} µg/(cm^2·day) after the initial 67 days. This is much closer to the target value than compared to the warmer test-sites, and the release rate was above 1 µg/(cm^2·day) for the initial 22 days. The theoretical starch consumption after 67 days at this site approximates 9.7 % of the starch initially available in the coating. It is emphasised that extrapolating this far from laboratory data is

uncertain; however the results seem to indicate the cause of the difference of antifouling effect between the test-sites.

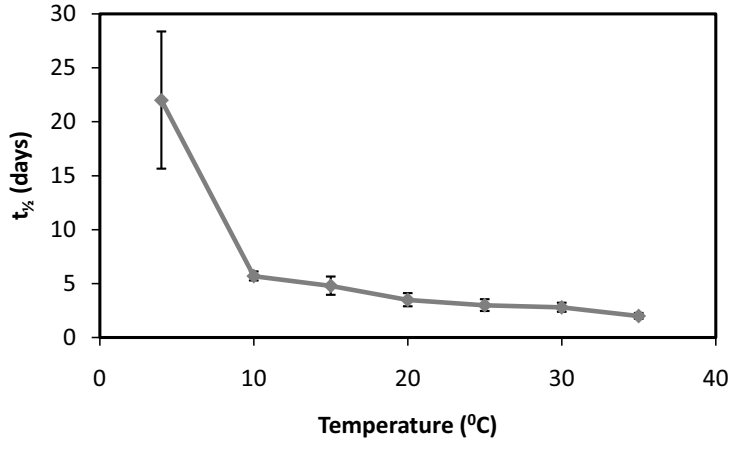

Figure 5.10: Development of half-life for hydrogen peroxide release rates for coating C as a function of temperature based on laboratory measurements in artificial seawater. Error bars are calculated as 95% confidence interval.

The results reported from the North Sea indicate that coating C provides some biofouling protection. The predicted release rate at the point of inspection is very low compared to the target, and therefore, it might be expected that the antifouling activity of the coating had seized within the initial 67 days. The results seen in Figure 5.8 would then have been caused by a delay of the onset of biofouling, and the colder climate would cause biofouling to occur more slowly than on the panels immersed in the Mediterranean and Indian Ocean. A schematic illustration of this hypothesised mechanism is provided in Figure 5.11. According to Egan (1987) biofouling progresses approximately 3.5 times more rapidly in seawater at 35 ºC than in seawater at 15 ºC, and this value has been used in the making of Figure 5.11. The figure illustrates that a delay in the onset of biofouling corresponding to the point in time where the theoretical release rate of hydrogen peroxide fell to below 1 µg/cm^2·day (22 days for the North Sea and 11 days for The Indian Ocean, see above), may account for the difference in antifouling of the same coating reported from two sites differing in climatic conditions. The release rate at the time of inspection can therefore not be used as a cursor for the hydrogen peroxide release needed to obtain antifouling properties. Looking at the point of inspection in the North Sea, it is seen that coating C can have less biofouling than

coating I, due to chemical activity that seized more than a month before the inspection. It is emphasized that the figure is based on somewhat hypothetical estimations and not on established data.

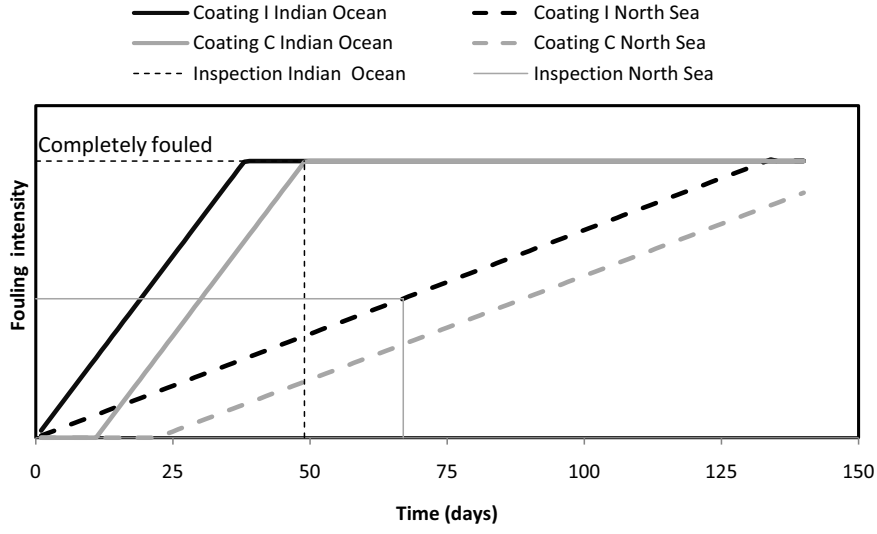

Figure 5.11: Qualitative illustration of the development of biofouling on the two blanks (coating I) and coating C panels used during raft tests in The Indian Ocean and The North Sea. A doubling of biofouling rate per 10 ºC temperature increase has been very crudely assumed.

From the above description of antifouling efficiency of the coatings, it can be concluded that the starch/enzyme system of the form used here is insufficient for antifouling purposes under warmer conditions. The primary reason for the difference in activity between what is reported here, and what was reported by Kristensen et al. (2009b) is the increased temperature. However, in order to be able to improve the system so that its potential can be fully uncovered, it is necessary to identify the part of the hydrogen peroxide-releasing system that fails over time.

The release rate measurements of coating C in artificial seawater gave very different results, when glucose was added to the water. The hydrogen peroxide concentrations increased rapidly (quantification not possible due to overload), which is shown in Figure 5.12. This indicates that the formation of glucose, due to hydrolysis of starch by glucoamylase, is the stability-limiting step of the coatings. However, as reported in (Kristensen et al. 2009a) stability of the glucoamylase in artificial seawater alone is greatly exceeding the stability of glucose formation in coating C reported in Figure 5.9. Therefore, simple glucoamylase decay is not the cause of activity loss. Furthermore, calculations of the amount of starch consumed during seawater immersions revealed that more than

90 vol% of the initial amount of starch was left at the point of inspection (see calculations above). It is therefore unlikely that starch depletion should be the cause of loss in release rate.

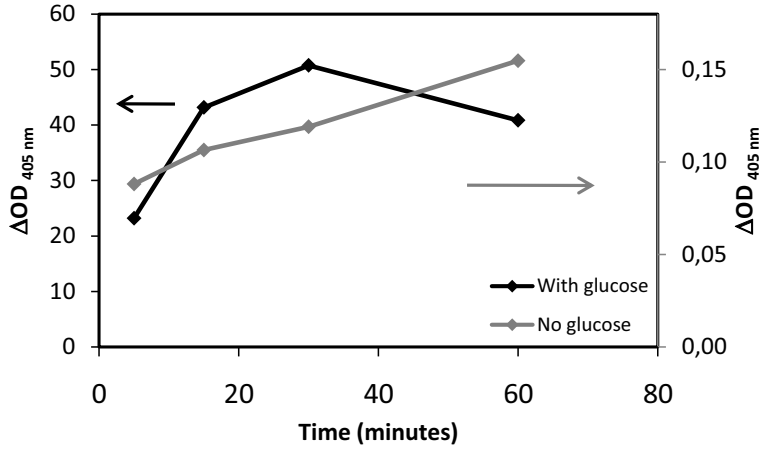

Figure 5.12: Comparison of the formation rate of hydrogen peroxide from coating C after 21 days in artificial seawater at 20 ºC. The black values are for a coating in glucose enriched (50.6 mM) artificial seawater, and the grey values are for a coating in glucose deficient water. Note the different scales on the y-axis. ΔOD is the absorbance of light at the given wavelength (405 nm).

The result of the leaching experiment using a rotary set-up (see Kiil et al. (2001) for apparatus details) for the C and H coating is shown in Figure 5.13. It is seen that the leaching of coating C ends relatively fast at a leached layer depth of approximately 20 µm. The uncertainty of the measurement is relatively high. However, Coating H leaches for a significantly longer time, and to a greater extent than coating C. The glucoamylase content of the two coatings should be identical, and therefore the only difference between the two coatings is the presence of hexose oxidase, leading to hydrogen peroxide generation. The results provided in Figure 5.13 indicate that the production of hydrogen peroxide in coating C gives rise to the rapid loss of glucoamylase activity, which means that the hydrogen peroxide produced by the second enzyme is distributed throughout the leached layer and not only released to the seawater as proposed above. It is therefore concluded that the bottleneck of the system is glucoamylase stability in the presence of hydrogen peroxide. Measures to improve antifouling performance of the coating described here should therefore foremost be concerned with the optimisation of the enzyme formulations. Polishing of the coatings would also improve antifouling activity of the coating because the enzyme activity hidden in the un-

wetted part of the coating (see above) will be activated, however, if the glucoamylase cannot withstand hydrogen peroxide this effect will be short lived.

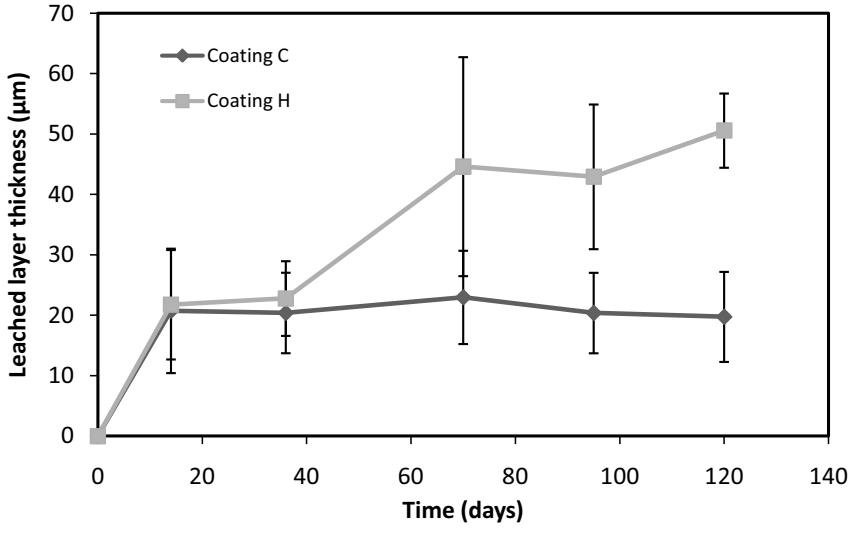

Figure 5.13: Development of leached layer thickness in coating C and H (no hexose oxidase present) during 4 month exposure to artificial seawater on a rotary system. Error bars indicate the 95 % confidence level calculated from several measurements of the leached layer thickness on each sample.

Conclusions

In this work, an antifouling coating based on enzymatic conversion of starch into hydrogen peroxide is tested. Coatings have been formulated and they retain the requirements towards homogeneity and mechanical stability required of an antifouling coating. However, the enzyme-mediated hydrogen peroxide release achieved is insufficient in terms of antifouling efficiency when tested under equatorial and Mediterranean climatic conditions. Under temperate (17 ºC) climatic conditions with corresponding low biofouling intensity, the coatings have earlier been described to provide antifouling protection. The difference in antifouling effect between the different types of climates can be explained by the temperature difference. The stability of the release rates of hydrogen peroxide from the coatings is in laboratory assays shown to be highly dependent on temperature, the higher the temperature, the more rapidly the hydrogen peroxide release rate drops. Furthermore, the loss of hydrogen peroxide release rate is traced back to a loss in glucose release by glucoamylase. However, neither starch depletion, nor glucoamylase stability of the native enzyme

in hydrogen peroxide free environment can explain the rapid decrease in activity. Leaching assays based on rotary testing show that glucoamylase activity seizes more rapidly when hexose oxidase is present. Hydrogen peroxide react to deactivate the glucoamylase and a stable system can only be obtained if glucoamyase are protected against hydrogen peroxide. First then, will a true picture of the long-term antifouling potential under heavy biofouling conditions be obtainable.

References

de Oliveira-Filho, E. C., Lopes, R. M., Paumgartten, F. J. R. 2004. Comparative study on the susceptibility of freshwater species to copper-based pesticides. Chemosphere **56**. p 369-374.

Dunn-Colemann, N., Neefe-Kruithof, P., Pilgrim, C., E., Ward, D., E., van Solingen, P. 2007. Trichoderma Reesei Glucoamylase and homologs thereof. US patent application US2007/0004018 .

Egan B. 1987. Marine microbial adhesion and its consequences. In Sleigh, M A, and Hoewood, H, Editors. *Microbes in the sea,* Ellis Horwood limited, Chichester p 220-238.

Elzanowska, H., Wolcott, R., G., Hannum, D., M., Hurst, J., K. 1995. Bactericidal properties of hydrogen peroxide and copper or iron-containing complex ions in relation to leukocyte function." Free radical biology & medicine **18,** (3). p 437-449.

Eul W., Moeller A., Steiner N. 2001. Hydrogen Peroxide. Kirk-Othmer Encyclopedia of Chemical Technology, p 1-53.

Grasshof K, Kremling K, Ehrhardt M. 1999. Methods of seawater analysis. Wiley-VCH Verlag GmbH & Co., Weinhwim Germany.

Hamade, R., Kadoma-dhi, Yamamori, Kyotanabe-shi. 1998. Method for controlled release of compounds having antimicrobial activity, and coating composition. US patent 6150146.

Ikuta, S., Ichikawa, S., Wakao, Y., Nishimura, K., Yasunaga, T. 1988. Inorganic peroxides for heat exchanger biofouling control. The American society of mechanical engineers.

Kiil, S., Dam-Johansen, K., Weinell, C., E., Pedersen, M., S. 2001. Analysis of self-polishing antifouling paints using rotary experiments and mathematical modelling. Ind. Eng. Chem. Res. **40**, 3906-3920.

Kiil, S., Dam-Johansen, K., Weinell, C., E., Pedersen, M., S. 2002. Seawater-soluble pigments and their potential use in self-polishing antifouling paints: simulation-based screening tool." Progress in organic chemistry **45,** 423-434.

Kiil, S, Dam-Johansen, K. 2007. Charaterization of pigment-leached antifouling coatings using BET surface area measurements and mercury porosimetry. Progress in Organic Coatings **60** (3). 238-247.

Kiil, S, Weinell, C E, Yebra, D M, Dam-Johansen, K. 2006. Marine biofouling protection: design of controlled release antifouling paints. In: Ng, K M, Gani, R, Dam-Johansen, K (ed) Chemical Product Design; Towards a Perspective Through Case Studies, 23IDBN-13: 978-0-444-52217-7. part II (7), Elsevier.

Kristensen, J., B., Meyer, R., L., Laursen, B., S., Shipovskov, S., Besenbacher, F., Poulsen, C., H. 2008. Antifouling enzymes and the biovhemistry of marine settlement. Biotechnology advances **26** 471-481.

Kristensen, J., B., Meyer, R., L., Poulsen, C., H., Shipovskov, S., Besenbacher, F., Laursen, B., S. 2009a. An oxidase-based catalytic system using biomimetic silica encapsulation for replacement of metal biocides in antifouling coatings. Green chemistry (yet unpublished, see enclosures 1 for paper manuscript).

Kristensen, J B, Olsen, S M, Laursen, B S, Poulsen, C H, Kragh, K M, Besenbacher, F, Meyer, R L. 2009b. The antimicrobial and antifouling activity of hydrogen peroxide in marine applications. Biofouling (Paper not yet published see enclosure 2 for paper manuscript)

Morris, R., S., Walsh, I., V. 1996. Zinc oxide photoactive antifoulant material. US patent 5,916,947.

Nishimura, K,. Yasunaga, T., Ichikawa, S., Wakao, Y. 1988. Development of a new antifouling method for a marine cooling water system. Marine biology **99,** 145-150.

Olsen, S., M., Pedersen, L., T., Laursen, M., H., Kiil, S., Dam-Johansen, K. 2007. Enzyme-based antifouling coatings: a review. Biofouling **23** (5). 369-383.

Olsen, S., M., Pedersen, L., T., Hermann, M., H., Kiil, S., Dam-Johansen, K. 2008. Inorganic precursor peroxides for antifouling coatings. JCTR (Paper available electronically).

Poulsen, C., H., Kragh, K., M. 1999. HOx as antifouling. US patent application US2002/0166237.

Sauer J, Sigurskjold B W, Christensen U, Frandsen T P, Mirgorodskaya E, Harrison M, Roepstorff P, Svensson B. 2000. Review Glucoamylase: structure/function relationships, and protein engineering. Biochemica et Biophysica Acta. **1542**. p 275-293.

Savary, B., Hicks, K., O'Connor, J. 2001. Hexose oxidase from Chondrus crispus: improved purification using perfusion chromatography. Enzyme and Microbial Technology, **29**, 42-51.

Perez, R., Noguera, D., Anderson, M. 2008. Localized generation of reactive oxygen species by pulsed electric signals and their effect on biological adhesion. Poster on the 14th international congress on marine corrosion and fouling, Kobe, Japan.

Perez-Roa, R., E., Anderson M., A., Rittschof, D., Orihuela, B., Wendt, D., Kowalke, G., L., Noguera, D., R. 2008. Inhibition of barnacle (*Amphibalanus amphitrite*) cyprid settlement by means of localized, pulsed electric fields. Biofouling **24**(3), 177-184.

Yebra, D M, Kiil, S, Weinell, C E, Dam-Johansen, K. 2006a. Parametric study of tin-free antifouling model paint behaviour using rotary experiments. Ind. Eng. Chem. Res. Paper **45**. P 1636-1649.

Yebra, D M, Kiil, S, Weinell, C E, Dam-Johansen, K. 2006b. Dissolution rate measurements of sea water-soluble pigments for antifouling paints: ZnO. Progress in Organic Coatings **56**. 327.-337.

Zhang, X., Yang, Z., Cai, Z. 2008. The hydrogen peroxide impact on larval settlement and metamorphosis of abalone Haliotis diversicolor supertexta. Chinese journal of oceanology and limnology **26**. p 238-241.

6. Chapter six - Investigation of antifouling effect of hydrogen peroxide flux

This chapter concerns the estimation of the required release rate of hydrogen peroxide for sufficient biofouling protection. A new test method will be described, depending on the release of biocide due to a gradient over a membrane. The test method has been used in laboratory assays on barnacle cyprids, and it has been adapted to seawater immersion for longer periods.

The work described in this chapter relies very much on the membrane design by Associate Professor Gunnar Jonsson at the Technical University of Denmark, which is greatly appreciated. Also, M.Sc. Student Inesa Tautkeviciute is thanked for her thorough report.

The content of this chapter is intended for publishing in the journal biofouling during 2009. The title will be 'Investigation of antifouling effect of hydrogen peroxide flux using novel laboratory and seawater trials.' (Authors: Olsen S M, Pedersen L T, Dam-Johansen K, Kiil S)

Background

Hydrogen peroxide (H_2O_2) is a potentially very attractive antifouling agent because it is unstable and decomposes to water and oxygen, as shown in equation (6.1):

$$2H_2O_2(aq) \rightarrow H_2O(l) + O_2(aq) \quad (6.1)$$

Decomposition of hydrogen peroxide is catalysed homogeneously by amongst others, catalase and iodine ions (Eul et al. 2001). Heterogeneous catalysis occurs with silver oxide for example (Eul et al. 2001), and photolytic catalysis takes place with UV light (Liao and Gurol 1995). Evidence points towards a free radical intermediate, implying that a chain reaction is involved (Eul et al. 2001):

$$H_2O_2\,(aq) \rightarrow 2HO\cdot(aq) \quad (6.2)$$

Hydrogen peroxide alone is a strong oxidant, but the hydroxyl radical is one of the most powerful oxidants known (Eul et al. 2001). Hydrogen peroxide is used to control pollution in waste waters (Eul et al. 2001). At elevated concentrations (1-3 wt%) it is used as a disinfectant in households and industry (Eul et al. 2001). In the US, hydrogen peroxide is 'generally recognized as safe' (GRAS), which means that it is considered a chemical safe to humans by the US Food and Drug Administration (Eul et al. 2001).

Hydrogen peroxide is present in nature, and can be regarded as nature's own disinfectant (Block 2000). It is known to function as a preservative in milk and honey, and it also exists in humans where it protects against infections. It is also found in the North Atlantic in a concentration of 0.14 µg/l and in Cheasapeake Bay in a concentration of 58 µg/l (Stringer 2001). Hydrogen peroxide also occurs in living animals, and is produced from metabolites released from the organisms (Stringer 2001).

In seawater, the half-life of hydrogen peroxide varies depending on location. Half-lifes range from less than 10 hours in coastal regions to 120 hours at surface waters in the open sea (Petasne and Zika 1997). Biologically mediated hydrogen peroxide decomposition by microorganisms is claimed to predominate (Petasne and Zika 1997). However, a catalytic effect of naturally occurring iodine ions on decomposition of hydrogen peroxide has been reported (Wong and Zhang 2008). The fate of hydrogen peroxide in soil depends on microbiological flora and the presence of minerals in the soil. However, in all cases, the half-life in soil is reported in minutes (Stringer 2001).

Hydrogen peroxide is an intermediate in oxygen metabolism, and to control the level of hydrogen peroxide produced in them, cells are equipped with catalase (Petasne and Zika 1997). Catalase is an enzyme that very rapidly degrades hydrogen peroxide to water and oxygen, and its presence in cells ensures that bioaccumulation of hydrogen peroxide does not occur (Stringer 2001). Marine plankton and bacteria have been shown to contain catalase, and hydrogen peroxide decomposition in coastal water has been shown to predominantly be caused by these enzymes (Petasne and Zika 1997).

The effects of hydrogen peroxide as antifouling compound reported in peer reviewed literature are summarised in Table 1.12 (see appendix I for elaborate discussion). It is evident that hydrogen peroxide does affect a wide range of biofouling species. However, it is also obvious that some are more robust towards hydrogen peroxide than others, which can be explained by the fact that biology has provided versatile means for organisms to protect themselves against hydrogen peroxide. Interestingly, there seems to be agreement that the effect of hydrogen peroxide as an antifouling agent is improved if catalysts, promoting its decomposition into reactive radicals are applied as well (Perez et al. 2008), (Ikuta et al. 1988), and (Nishimura et al. 1988). For an more thorough discussion on hydrogen peroxide in seawater and its antifouling effect, the reader is referred to Appendix I.

Hydrogen peroxide release from marine coatings can be obtained using enzymes and starch (Poulsen and Kragh 2002), precursor peroxides (Olsen et al. 2008) or photocatalytic active pigments (Morris and Walsh 1999). All of the effects of hydrogen peroxide and its reactive derivatives reported have been based on bulk solution concentration studies or coating-based studies. It is important to distinguish between results obtained from assays with the organisms present in a solution of the active compound, and the potential effect of a flux in µg/(cm^2·day) of the compound. What biofouling animals at or very near a coated ship hull will experience is a concentration gradient of active ingredient across a laminar layer of seawater (approximately 0.1 mm wide according to the film theory and dependent on the hydrodynamic conditions Levenspiel, (1999). At the very coating surface, the concentration will be highest and dictated by release rates of hydrogen peroxide from the coating (Kiil et al. 2002). At the other side of the laminar diffusion layer, the biocide concentration will be zero. At positions within the laminar layer, the biocide concentration will be somewhere in between these two boundary values. Therefore, an organism will have to move up the biocide gradient prior to settlement. However, depending on the size and morphology of the biofouling organism, it may also experience biocide-free seawater at the end of the organism pointing away from the coating (organisms larger than the thickness of the laminar film). Also, the organism can choose to move away from the high biocide concentration. The difference between what is experienced by a relatively large and a relatively small biofouling organism during the initial approach to the coating surface is schematically illustrated in Figure 6.1. Consequently, exposing a biofouling organism to a fluc of biocide is quite different to exposing it to a bulk biocide concentration.

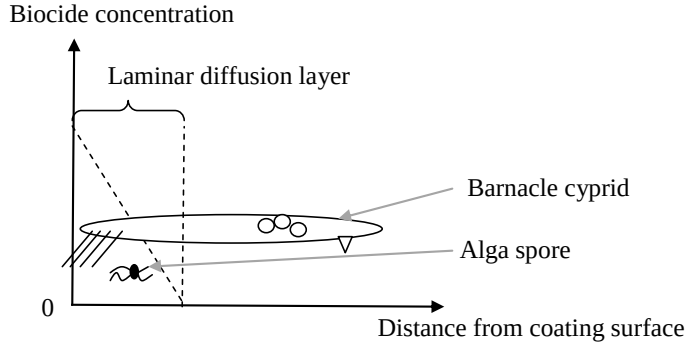

Figure 6.1: Schematic illustration of how two different-sized biofouling organisms may experience a flux of biocide from an antifouling coating. The laminar diffusion layer is about 100 µm wide, but the thickness is dependent on sailing speed. Figure approximately to scale.

Important engineering questions to be answered when evaluating biocides are: Will the required amount of active ingredient be technically feasible in a film thickness of between 150 and 300 µm? Will the critical pigment volume concentration be exceeded? Are the amounts of biocide or precursor needed compatible with the binder? It is obvious that these questions cannot be answered based on effective bulk concentrations of biocide. Consequently, in this work a new method of testing antifouling agents has been designed. Controlled release rates of hydrogen peroxides are achieved using cellulose acetate membranes, and biofouling resistance assays, based on release in both laboratory and seawater, have been carried out.

Previous biofouling resistance testing

The most common laboratory test for antifouling agents is the barnacle (*Balanus amphitrite*) test first described by Rittschof et al. (1992) and recently improved by Qiu et al. (2008). The test is based on the effective concentrations of the biocide in the bulk solution towards settlement, metamorphosis or death of barnacle larvae kept in a Petri dish or other artificial laboratory set-ups. The results are typically presented in terms of EC_{50} or LC_{50} values. The former indicates the effective concentration hindering settlement or metamorphosis by 50 % of the individuals, and the latter describes the lethal concentration for 50% of the individuals. This method has become very popular. It is cheap, simple to use, and relatively inexpensive. However, though the method has become a great success in evaluating new biocides, it has shortcomings when it comes to formulation of antifouling coatings. The method provides information on the relative potency of a biocide, but it is not possible to identify the amount of biocide needed in a coating formulation because a chemically active antifouling coating provides a flux of biocide and not a bulk concentration. An alternative to testing potential antifouling agents in bulk solution is to have them enclosed in a coating matrix, and then test the antifouling ability of the coating. The coating can be applied on panels and exposed to seawater, or directly on ship hulls, or it can be tested in laboratory assays similar to the barnacle test. However, this is an indirect method because even if the amount of biocide released is estimated one cannot be certain that the release rate has been constant throughout the exposure time.

In addition to laboratory assays involving larvae of barnacles, adult barnacles are used to test for fouling release properties of a coating in reattachment studies (Rittschof et al. 2008). Settlement behaviour of the zoospore of the algae *Ulva linza* has been subject to exhaustive

research (Callow et al. 1997; Callow and Callow 2006). The research has resulted in laboratory assays where the algae spores are used to test antifouling agents for settlement prevention (Callow et al. 1997). Also, the spores' adhesion strength is used in laboratory assays to test the fouling release properties of surfaces (Granhag et al. 2004), and ultimately the detailed knowledge of settlement behaviour of *Ulva linza* obtained has resulted in alternative approaches to fouling prevention (Ederth et al.2008). Diatoms settlement behaviour has also been extensively studied (Thompson et al. 2008) and adhesion strength of diatoms to surfaces is also used in laboratory fouling release assays (Pettitt et al. 2004). Furthermore, marine bacteria in their planktonic stage are used to screen for antifouling agents, and biofilm formation of the bacteria are used as a cursor for antifouling coating potency (Kristensen et al. 2009).

Though several other advanced alternatives to testing antifouling agents have been described, such as casting active agents in agar plates (McLean et al. 2004), test for inhibition of enzymes responsible for curing adhesive (Hellio et al. 2000), and monitoring of motion behaviour of algal spores (Iken et al. 2003), it has not been possible to find a reference describing antifouling tests based on well-established release rates of the biocide.

Strategy of investigation

The aim of this paper is twofold. To establish the usefulness of hydrogen peroxide-based antifouling, a novel test method has been developed. The controlled release set-up described has been prepared based on a membrane separating a biocide solution from the test chamber. The relationship between gradient of biocide over the membrane and biocide release rate from the membrane has been established.

Testing of the impact of different hydrogen peroxide release rates on barnacle larvae settlement has been carried out in the laboratory, and the results have been compared to the effect of bulk solution concentration of hydrogen peroxide on barnacle larvae. In addition, ocean experiments of hydrogen peroxide release rates have been performed to test the release rate of hydrogen peroxide required to obtain efficient antifouling properties under natural conditions.

Experimental – Materials and methods

Membranes

Several types of membranes of different thicknesses were tested for their applicability: Clay membranes, ceramic membranes, and cellulose acetate membranes. The porous clay and ceramic membranes were disregarded due to catalytic effects on hydrogen peroxide decomposition. Consequently, the membranes applied in the experiments were made of cellulose acetate (Cas nr 9004-35-7). They were prepared from a solution of 80 wt% butanone (commercial grade) and 20 wt% cellulose acetate. Dr Blade applicators (500 µm – 1000 µm gap sizes) were used to prepare the membranes. The first set of membranes, to be used in the laboratory setup was prepared, as free films on glass plates. After drying, the membranes were easily removed from the substrate by water-immersion, whereby the membrane was loosened from its substrate and easily removed. The second set of membranes, to be used for ocean experiments, was prepared on Kraft paper with a thickness of 75 µm. Kraft paper is made of wood pulp and has been used by others as a carrier for films when measuring permeability (Carneiro et al. 2006). Kraft paper was used here to improve surface properties of the test membranes, allowing for biofouling accumulation, without adding to the diffusion resistance of the membrane. The thickness of the dry cellulose acetate membranes varied between 40 µm and 100 µm.

Ocean testing of the membranes

To test the tendency of biofouling to settle on cellulose acetate membranes in the absence of hydrogen peroxide release, the membranes were exposed to natural conditions in Singapore where the biofouling intensity is high. A blank acrylic panel and one panel painted with an inert coating (Hempels Hempatex high-build 4633) were used as references, and biofouling was allowed to accumulate for seven weeks before inspection.

Generation and control of biocide release rates

Effective release rates of hydrogen peroxide were measured using the chamber set-up depicted in Figure 6.2. The apparatus consists of a 10 cm · 10 cm · 10 cm^3 middle chamber. Two smaller chambers, with a diameter of 4 cm and a length of 6 cm, are attached to the centre chamber,

allowing for duplicate measurements. In-between the middle chamber frame, and the two smaller chamber frames, membranes were mounted, separating the solutions in the chambers.

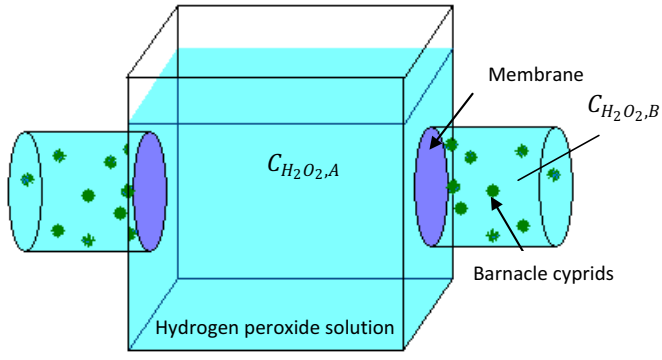

Figure 6.2: Schematic illustration of the chamber setup used to test release rates of hydrogen peroxide against barnacles. C(H_2O_2,A) is the hydrogen peroxide concentration in the centre chamber at time t, and C(H_2O_2,B) the hydrogen peroxide concentration in the barnacle chambers at time t.

The relationship between the gradient over the membrane and the flux through the membrane was established before barnacle testing was initiated. Theoretically, the relationship between flux and concentration can be described by equation (6.3), which is derived from Fick's first law of diffusion:

$$J = \frac{k \cdot D}{l}\left(C_{H_2O_2,C} - C_{H_2O_2,B}\right) \approx \frac{k \cdot D}{l} C_{centre} \qquad (6.3)$$

J is the flux through the membrane, *k* an empirical factor relating the diffusivity in the membrane to the bulk water diffusivity of the compound, and *l* the thickness of the membrane. $C_{H_2O_2,A}$ denotes the hydrogen peroxide concentration in the centre chamber, and $C_{H_2O_2,B}$ the hydrogen peroxide concentration in the chamber with barnacles.

The flux of hydrogen peroxide through the membrane was measured via the increase of hydrogen peroxide concentration in the constant volume of water in the barnacle chambers. 775 ml of a solution of known concentration of hydrogen peroxide (0.5 mM to 50 mM) in deionised water was added to the centre chamber, and 75 ml of diluted hydrogen peroxide (~0.002 mM) was added to the small chambers to ensure that hydrogen peroxide concentrations were above the detection limit (~0.001 mM) of the assay from start of the measurements. This was done simply to reduce the time required for the solution in the chamber to reach detectable levels. Hydrogen peroxide

concentration was determined spectrophotometrically using a coupled enzyme assay based on 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). ABTS reacts with hydrogen peroxide in a peroxidase-catalysed reaction (peroxidase is therefore also added), and hydrogen peroxide concentrations can thereby be indirectly determined by measuring the absorption by oxidised ABTS at 405 nm in an assay adapted from (Savary et al. 2001). After stirring, 50 µl of solution was frequently sampled from the small chambers. The liquid content of the small chambers did not decrease to less than 99.6 % of the original value due to sampling, and therefore the volume was considered constant during the experiment (potential volume change due to diffusion of hydrogen peroxide is neglected). The sample was mixed with 250 µl of a solution containing ABTS and peroxidase, and a calibration curve was obtained to convert absorbance to hydrogen peroxide concentrations.

Barnacle larvae settlement behaviour in release rate assay under laboratory conditions

Cyprids of the barnacle species *Balanus improvisus* and filtered brackish water (~1.5 wt % salinity) was retrieved from Tjärnö Marine laboratories in Sweden. Filtered brackish water was added to the small chambers of the apparatus (cf. Figure 6.2), and a hydrogen peroxide solution in deionised water was added to the centre chamber. Barnacle larvae were transferred to the barnacle chambers using a small piece of cloth mounted to a spatula. Settlement behaviour of the barnacles was monitored over three days. The larvae were reluctant to settle (did not) on the membrane material, which is probably due to the firm less hydrophilic alternative provided by the polycarbonate chamber wall. As a consequence, settlement pattern of the barnacle larvae in the small chamber was monitored instead of barnacle settlement on the actual membrane. The chamber was divided into 12 equally sized areas, and the number of barnacles settled within each area was counted by visual inspection.

Different fluxes of hydrogen peroxide through the membranes were tested on barnacle settlement pattern. The release rates tested included 0, 5, 8, and 40 µg/(cm^2·day) of hydrogen peroxide.

Bulk hydrogen peroxide concentration assay

Larvae of the species *Balanus improvisus* were retrieved from Tjärnö, Sweden. Brackish water (1.5 wt % salinity) was withdrawn at Bellevue (north of Copenhagen, Denmark). Seven hydrogen

peroxide solutions were prepared using filtered seawater. The concentrations ranged from 0.1 µM (3.4 µg/l) to 0.1 M (3.4 g/l) with tenfold increase in concentration from one solution to the next. Five ml of hydrogen peroxide dilution in filtered seawater was added to a Petri dish, and barnacle larvae were added using a small cloth mounted on a spatula stick. The Petri dishes were kept together to ensure identical temperatures. Larvae activity was then monitored for a week, with daily evaluations. As the larvae did not settle, only activity and death were observed.

Release rate assay in seawater (ocean testing)

Seawater trials were performed in 10 litre plastic containers. The centre part of the lids of the tanks was drilled out, and membranes were mounted in the lid void with an additional gasket underneath. A schematic drawing of the tank used for seawater trials is shown in Figure 6.3.

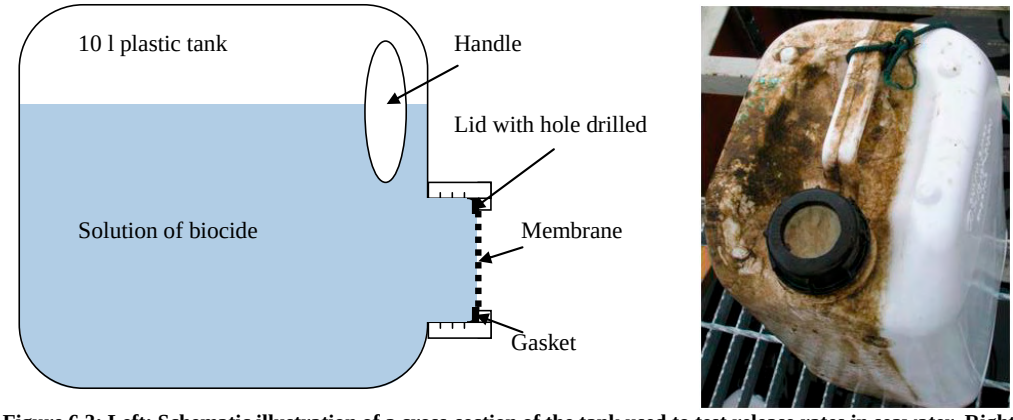

Figure 6.3: Left: Schematic illustration of a cross-section of the tank used to test release rates in seawater. Right: picture of a tank taken after 6 weeks in the sea.

Ten kilograms of hydrogen peroxide solutions were produced. The solutions contained hydrogen peroxide of a concentration of approximately 0, 5 and 50 mM. The membranes were of a thickness of 40 µm attached to Kraft paper 75 µm thick and attached with the Kraft paper facing outward, to hide a potential biofouling deterring effect of the cellulose acetate membrane. The Kraft paper was sufficiently porous not to provide any diffusion resistance to water or biocide. Two tanks releasing hydrogen peroxide with a rate of approximately 224 and 2800 (µg/cm^2·day) were prepared. In addition, a control containing only water was included in the series.

The containers were immersed in seawater in Jyllinge harbour, Denmark. They were secured in a horizontal position using a rope, and the small volume of air still in the container kept them near the surface. The water salinity was ~1.5 wt%, and the temperature was around 14 ºC during the experiment. The test began early September 2008 and ended November 2008, and the membranes were inspected every 14 days. To ensure stable release rates from the membranes, the hydrogen peroxide concentrations in the tanks were monitored semi quantitatively using Merckoquant® peroxide test from Merck with a detection range from 0.5 to 25 mg/l. Dilution of the tank contents by a factor of 17 for the low concentration tank and 170 for the high concentration was done using deionised water prior to measurement. After a 2 months immersion period, the hydrogen peroxide content of the tanks was replenished.

Results

The results of the various tests conducted will now be discussed.

Ocean testing of the membranes

Figure 6.4 shows the biofouling accumulated on a cellulose acetate membrane (no hydrogen peroxide flux) compared to blank panels after 7 weeks immersion in seawater in Singapore. It is evident that the three materials are equally fouled, and the composition of the biofouling was identical on the three materials. Thus, in ocean waters cellulose acetate membranes certainly foul.

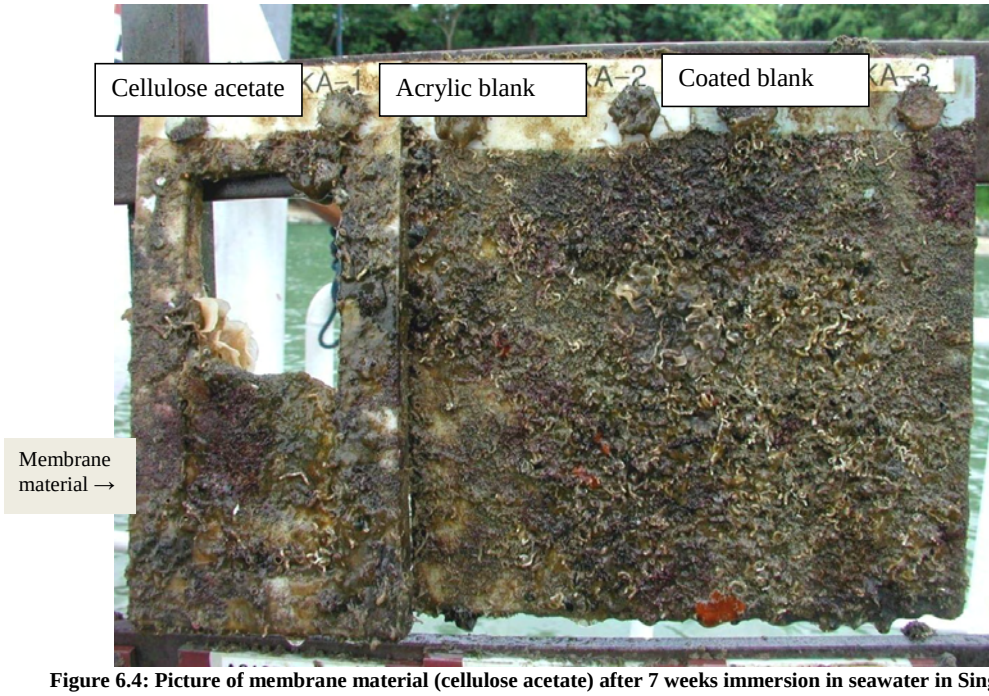

Figure 6.4: Picture of membrane material (cellulose acetate) after 7 weeks immersion in seawater in Singapore. From left to right: Cellulose acetate (the membrane was broke and only the bottom part is left on the picture), acrylic blank, and a control blank coated with inert coating.

Generation and control of biocide release rate

The measurements of concentration increase in the small chambers of the test apparatus showed a linear trend, indicating a stable release rate. The fluxes achieved in the membrane experiment span from 25 to 2800 µg/(cm^2·day) corresponding to 0.74 to 88 µmoles/(cm^2·day). The measurements for one of the membranes have been plotted in Figure 6.5, and the plot of the obtained fluxes as a function of centre chamber hydrogen peroxide concentration for all the data is shown in Figure 6.6. It is seen that there is an almost linear correlation between concentration in the centre chamber and the flux achieved through the membrane.

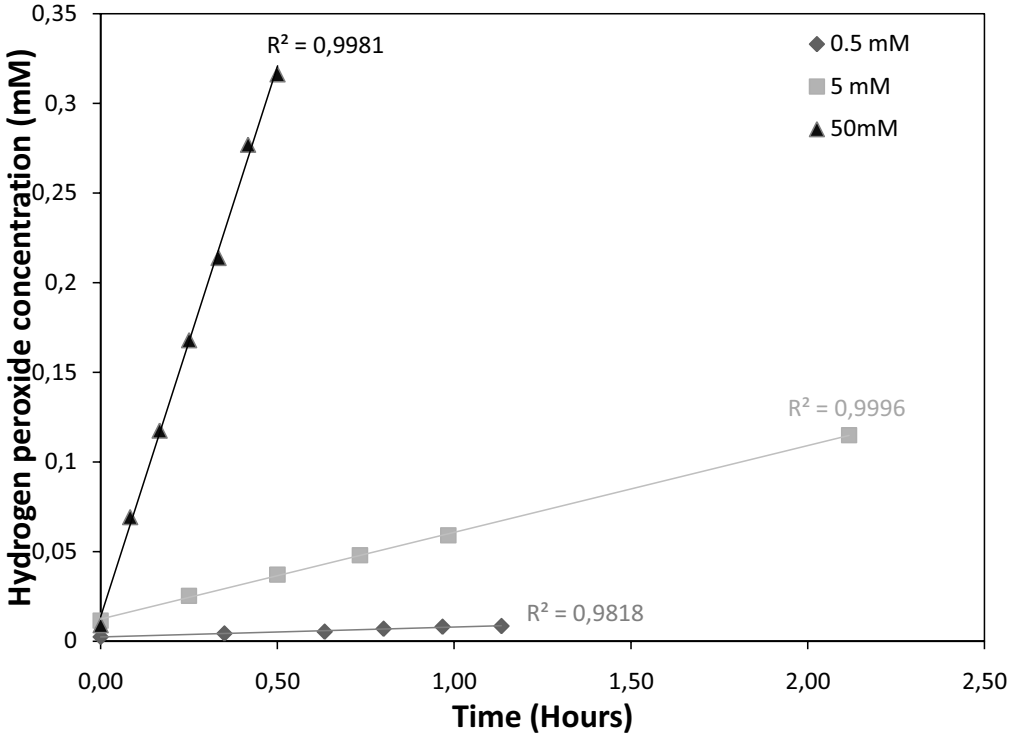

Figure 6.5: Increase in concentration of hydrogen peroxide in the small chamber as a function of time. Three hydrogen peroxide concentrations were tested in the centre chamber. The results for 0.5, 5 and 50 mM H_2O_2 are shown.

The slope of the curves can be used to calculate the effective diffusion coefficient of hydrogen peroxide in the membrane (the product k·D from equation (6.3)). This is done using the area (12.6 cm^2) and thickness of the membranes (40 µm), and a value of $7.7 \pm 1 \cdot 10^{-12}$ m^2/s is obtained. The diffusion coefficient of hydrogen peroxide in water is approximately $1.3 \cdot 10^{-9}$ m^2/s. The k-parameter from equation (6.3) is therefore approximately 0.006, meaning that the membrane material decreases the effective diffusion of hydrogen peroxide by a factor of 169.

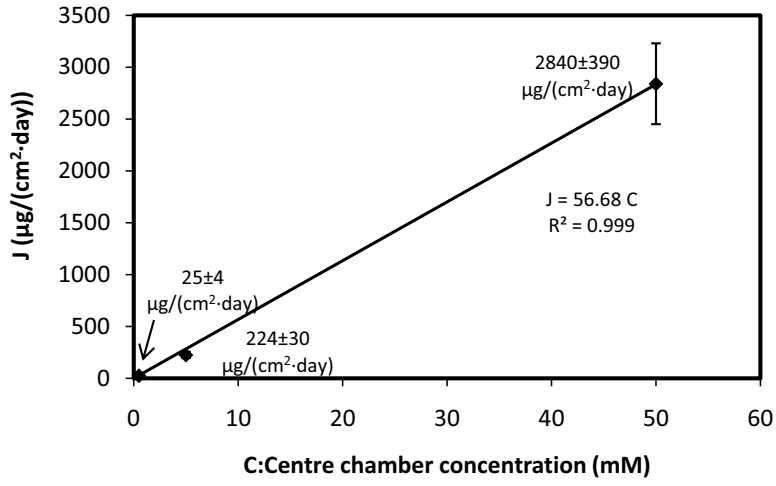

Figure 6.6: Fluxes measured as a function of centre chamber hydrogen peroxide concentrations. The plot is a result of measurements on two different membranes, and 95% confidence interval is indicated by the error bars. J is the hydrogen peroxide flux.

Barnacle larvae settlement behaviour in release rate assay under laboratory conditions

Figure 6.7 shows the percentage of settled larvae against the distance from the flux-generating membrane. An F-test performed on the variations of the different distributions showed that the barnacle distribution in the 40 µg/(cm^2·day) measurement is significantly (>95 % confidence level) different from the blank. However, for the lower fluxes (5 and 8 µg/(cm^2·day)), the barnacle distribution was not statistically different from the blank. The repeatability of the experiment is identified when comparing the two measurements using 40 µg/(cm^2·day). When the percent of larvae settled is plotted against the distance from the flux membrane as seen in Figure 6.8, the slope becomes 2.8±0.1. The error is calculated as a 95% confidence level and therefore good repeatability is achieved.

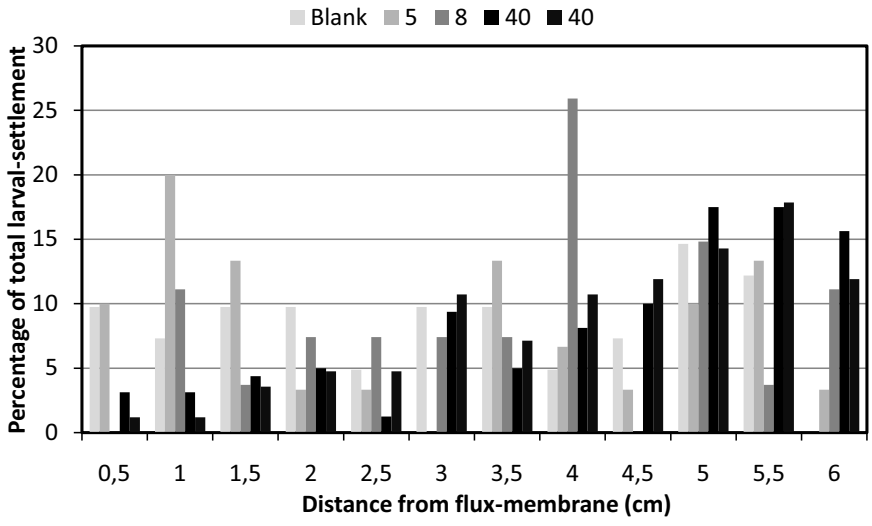

Figure 6.7: Settlement behaviour of barnacles in laboratory membrane assays. Flux in µg/(cm^2·day).

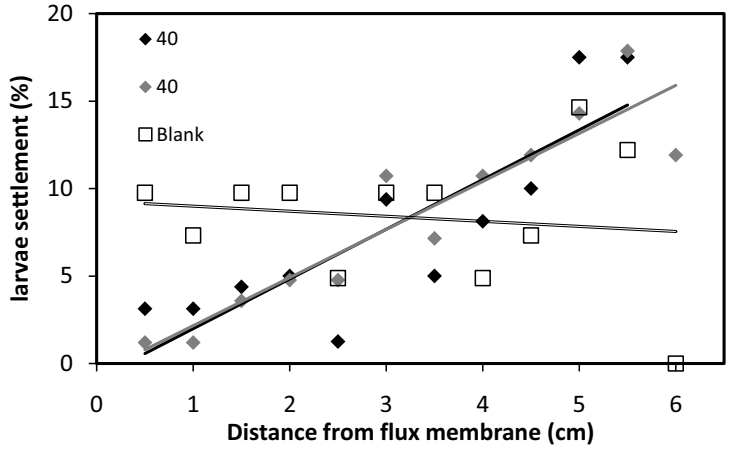

Figure 6.8: Comparison between the barnacle settlement pattern during the two 40 µg/(cm^2·day) measuements and the blank measurement.

Effect of bulk hydrogen peroxide concentration on barnacle cyprids

The barnacle cyprids transferred to the Petri dishes containing hydrogen peroxide solution did not settle. The hydrogen peroxide solution was not refreshed and therefore the effects of hydrogen peroxide is expected to have occurred within the first day. In
Table 6.1, the effect of hydrogen peroxide on the barnacles after one day has been summarised. It is seen that the only visible effect of the hydrogen peroxide solution was death of the larvae. Death was the result for the barnacle larvae exposed to concentrations of 3400, 340 and 34 mg/l (100 mM, 10 mM, 1 mM) hydrogen peroxide. However, barnacle larvae survived concentrations from 3.4 mg/l (0.1 mM) and downwards.

Table 6.1: Effect of bulk concentration hydrogen peroxide on cyprid larvae of the barnacle species *Balanus improvisus*.

Dilution #	Hydrogen peroxide concentration	Barnacle condition after 1 day
2	3400 mg/l (100 mM)	Dead (100%)
3	340 mg/l (10 mM)	Dead (100%)
4	34 mg/l (1 mM)	Dead (100%)
5	3.4 mg/l (0.1 mM)	Active
6	0.34 mg/l (10 µM)	Active
7	0.034 mg/l (1 µM)	Active
8	0.0034 mg/l (0.1 µM)	Active
Control	0	Active

Release rate assay in seawater

Figure 6.9 shows pictures taken of the three membranes after 14, 32, 46 and 67 days immersion in Jyllinge Harbour. Looking at the pictures, it is evident that whereas the membrane not releasing any hydrogen peroxide is considerably fouled by slime already after 14 days, both the membrane releasing hydrogen peroxide with a rate of approximately 224 µg/(cm^2·day) and that releasing approximately 2800 µg/(cm^2·day) are biofouling free. After 32 days, slime is beginning to show on the intermediate concentration membrane, this tendency is even more pronounced after 46 days. After 67 days the intermediate membrane is covered with less slime than after 46 days. However, this is also the case for the control.

Figure 6.9: Pictures of the flux membranes after 14 (bottom series), 32 (middle), 46 days, and 67 days immersion in Jyllinge Harbour. From left to right: reference membrane (control), 224 µg/(cm^2·day) and 2800 µg/(cm^2·day) hydrogen peroxide release rates.

In the place of slime, seaweed is now attached to the control and the intermediate concentration membrane (considerably more on the control). The seaweed was firmly attached to the membranes. The change in fouling type is caused by the change in the seasonal conditions. During this period (from October 27th to November 17th) the seawater temperature in Roskilde fjord (Jyllinge harbour) dropped from above 10 ºC to around 5 ºC, significantly altering the conditions for microbial growth. The general impression of the biofouling resistance of the three membranes is therefore the same for all sampling times. Note, that the Kraft papers are gradually bleached by hydrogen peroxide as time passes.

Discussion

The relationship between flux and gradient is linear as described by equation (6.3). The stability of the release rate over the membrane is therefore determined by the stability of the biocide concentration. If hydrogen peroxide degradation is neglected and only release through the membrane considered, 63 days are required before the flux is reduced to 90 % of its initial value for the membranes used in this experiment. This figure is independent on concentration and temperature (only flux is considered) and therefore only membrane-dependent.

The effect of hydrogen peroxide release rate on barnacle larvae settlement behaviour under laboratory conditions, as reported in this paper, is due to the release of hydrogen peroxide over the membrane in one end of the chamber, which causes a gradient of hydrogen peroxide in the barnacle chamber, and chemo taxis of the larvae allow them to select the low concentration end of the gradient.

The reluctance of barnacle larvae to settle on the membrane under laboratory conditions is very illustrative for the comparison between laboratory assays and seawater-based assays when testing the potential of new antifouling agents. From Figure 6.4, it is evident that biofouling occurs as rapidly on the membrane material (without a biocide flux) as on an inert blank when immersed to natural biofouling conditions. However, under no circumstances did the barnacle cyprids used in the assays described here settle on the membrane under laboratory conditions. Therefore, antifouling results obtained using a single biofouling species under laboratory conditions should be interpreted with caution. This is emphasised by the fact that a release rate of 40 µg/(cm^2·day) significantly affected barnacle settlement behaviour under laboratory conditions. However, between 50 and 500

times that amount is needed to keep Kraft paper biofouling-free when immersed in the sea under mild biofouling conditions during the fall in Denmark.

The seawater trials showed that a release rate of at least 224 μg/(cm^2·day) is needed to provide antifouling activity at an acceptable level in seawater. Two ways to provide hydrogen peroxide from an antifouling coating has been reported: enzyme based conversion of glucose or starch (Poulsen and Kragh 2002) and use of inorganic peroxides (Olsen et al. 2008). If 50 vol % of starch is used in a 300 μm thick antifouling coating, a release rate of 224 μg/(cm^2·day) can be kept for approximately eight days only, and if a similar amount of zinc peroxide, which is the only applicable antifouling coating ingredient reported in Olsen et al. (2008), is used, 37 days of a release rate of 224 μg/(cm^2·day) can be achieved. Therefore, it is highly unlikely that an antifouling coating will ever be able to provide its antifouling effect alone based on the release of hydrogen peroxide.

Fouling of the membranes in the seawater experiment will probably influence the release rate of biocide from the coating. However, this is what also occurs for antifouling coatings when immersed in the sea (Yebra et al. 2006).

The ocean test method that has been described here to obtain a controlled release of hydrogen peroxide may be applied to other biocides as well. The k-value from equation (6.3) is a membrane parameter and should therefore ideally apply to other biocides as well. Furthermore, should cellulose acetate prove insufficient, other membrane types can be applied. Ceramics and clay have been tested, but discarded due to the materials catalytic effect on hydrogen peroxide decomposition. However, for other biocides the hard material of these membranes may prove more suitable than the more flexible cellulose acetate membrane. Biofouling resistance of any biocide should be tested at natural seawater conditions.

Conclusions

This work provides a method of producing and controlling a flux of biocides through a surface for testing of antifouling potency. It is shown, using hydrogen peroxide, that the release rate from the membrane can be controlled by the biocide concentration in the container.

Using the novel test method, it has been established, that a release rate of hydrogen peroxide between 224 and 2800 μg/(cm^2·day) is needed to effectively prevent biofouling under the seawater conditions present in Denmark during the fall. This is between 50 and 500 times the release rate significantly affecting settlement of barnacle larvae in laboratory assays. These results indicate that

neither starch based release of hydrogen peroxide, nor inorganic peroxides will be able to deter biofouling for sufficiently long time when used as a mean for biofouling control alone.

Settlement behaviour of barnacle larvae under laboratory conditions is significantly altered at lower release rates, and when subject to bulk hydrogen peroxide concentrations, barnacle larvae are effectively killed at very low concentrations. It is therefore concluded, that to be able to evaluate the effect of a given antifouling biocide, testing should be brought to the natural marine environment. The test equipment described here allows for testing in seawater while knowing the release rate of the biocide tested.

References

Block, S S (editor). 2000. Disinfection, Sterilization and Preservation. Fifth edition. Lippincott Williams & Wilkins.

Callow M E, Callow J A. 2006. The *Ulva* spore adhesive system. In Smith, A M and Callow J A (Editors), Biological adhesives. Springer-Verlag, Berlin, Heidelberg. p 63-78.

Callow M E, Callow J A, Pickett-Heaps J D, Wetherbee R. 1997. Primary adhesion of *Enteromorpha* (Chlorophyta, ulvales) propagules: quantitative settlement studies and video microscopy. J. Phycol. **33**. p 938-947.

Carneiro C, Oliveira F, Nogueira J. 2006. Permeability of paint films towards chloride ion. JCT Research **3** (2). p 159-162.

de Oliveira-Filho E C, Lopes R M, Paumgartten F J R. 2004. Comparative study on the susceptibility of freshwater species to copper-based pesticides. Chemosphere **56**. p 369-374.

Ederth T, Nygren P, Pettitt M E,Östblom M, Du C-X, Broo K, Callow M E, Callow J, Liedberg B. 2008. Anomalous settlement behaviour of *Ulva linza* zoospores on cationic oligopeptide surfaces. Biofouling **24** (4). p 303-312.

Eul W., Moeller A., Steiner N. 2001. Hydrogen Peroxide. Kirk-Othmer Encyclopedia of Chemical Technology. p 1-53.

Granhag L M, Finlay J A, Jonsson P R, Callow J A, Callow M E. 2004. Roughness-dependent removal of settled spores of the green alga *Ulva* (syn. *Entermorpha*) exposed to hydrodynamic forces from a water jet. Biofouling **20** (2). p 117-122.

Hellio C, Bourgougnon, le Gal Y. 2000. Phenoloxidase from the byssus gland of Mytilus edulis: Purification, partial characterization and application for screening products with potential antifouling activities. Biofouling **16**. p 235-244.

Iken K, Greer, S P, Amsler, C D, McClintock, J B. 2003. A new antifouling bioassay monitoring brown algal spore swimming behaviour in the presence of Echinoderm extracts. Biofouling **19** (5). p 327-334.

Ikuta S, Ichikawa S, Wakao Y, Nishimura K, Yasunaga T. 1988. Inorganic peroxides for heat exchanger biofouling control. The American society of mechanical engineers.

Kiil S, Dam-Johansen K, Weinell C E, Pedersen M S. 2002. Seawater-soluble pigments and their potential use in self-polishing antifouling paints: simulation-based screening tool. Progress in organic coatings. **45**. p 423-434.

Kristensen J B, Olsen S M, Laursen B S, Poulsen C H, Kragh K M, Besenbacher F, Meyer R L. 2009. The antimicrobial and antifouling activity of hydrogen peroxide in marine applications. Biofouling. (Paper not accepted for publishing yet).

Levenspiel O. 1999. Chemical reaction engineering. Ind. Eng. Chem. Res. **38.** p 4140-4143.

Liao C-H, Gurol M D. 1995. Chemical oxidation by photolytic decomposition of hydrogen peroxide. Environ. Sci. Technol. **29**. p 3007-3014.

McLean R J C, Pierson III L S, Fuqua C. 2004. A simple screening protocol for the identification of quorum signal antagonists. Journal of microbiological methods **58**. p 351-360.

Morris S R, Walsh M A. 1999. Zinc oxide photoactive antifoulant material. USpatent 5,916,947.

Nishimura K, Yasunaga T, Ichikawa S, Wakao Y. 1988 “Development of a new antifouling method for a marine cooling water system.” Marine biology. **99**. Pp 145-150.

Olsen S M, Pedersen L T, Hermann M H, Kiil S, Dam-Johansen K. 2008. Inorganic precursor peroxides for antifouling coatins. Journal of coatings technology and research. (The paper has only been published electronically so far).

Perez R, Noguera D, Anderson M. 2008. Localized generation of reactive oxygen species by pulsed electric signals and their effect on biological adhesion. Poster on the 14th international congress on marine corrosion and biofouling.

Petasne R G, Zika R G. 1997. Hydrogen peroxide lifetimes in south florida coatsal and offshore waters. Marine Chemistry **56**. p 215-225.

Pettitt M E, Henry S L, Callow M E, Callow J A, Clare A S. 2004. Activity of commercial enzymes on settlement and adhesion of cypris larvae of the barnacle *Balanus amphitrite*, spores of the green alga *Ulva linza*, and the diatom *Navicula perminuta*. Biofouling **20** (6). p 299-311.

Poulsen C H, Kragh K M. 2002. Compostion. US patent application US2002/0106361A1.

Qiu J-W, Hung O S, Qian P-Y. 2008. An improved barnacle attachment inhibition assay. Biofouling **24** (4). p 259-266.

Rittschof D, Clare A S, Gerhart D J, Mary S A, Bonaventura J. 1992. Barnacle in vitro assays for biologically active substances: toxicity and settlement inhibition assays using mass cultured Balanus amphitrite amphitrite Darwin. Biofouling, **6**(2). p 115-122.

Rittschof D, Orhuela B, Stafslien S, Daniels J, Christianson D, Chisholm B, Holm E. 2008. Barnacle reattachment: a tool for studyiong barnacle adhesion. Biofoulinf **24** (1). p 1-9.

Savary B, Hicks K, O'Connor J. 2001. Hexose oxidase from Chondrus crispus: improved purification using perfusion chromatography. Enzyme and Microbial Technology, **29**, p 42-51.

Stauber J L, Florence T M. 1987. Mechanism of toxicity of ionic copper and copper complexes to algae. Marine biology 94 (511). p 511-519.

Stringer, D A, (Editor). 2001. Joint assessment of Commodity Chemicals No. 22 – Hydrogen peroxide CAS No. 7722-84-1. European Centre for Ecotoxicoloy and Toxicology of Chemicals.

Thompson S E M, Taylor A R, Brownlee C, Callow M E, Callow J A. 2008. The role of nitric oxide in diatom adhesion in relation to substratum properties. J phycol. **44**. p 967-976.

Wong, G T F, Zhang L-S. 2008. The kinetics of the reactions between iodide and hydrogen peroxide in seawater. Marine Chemistry **111**. Pp 22-29.

Yebra D M, Kiil S, Dam-Johansen K. 2004. Review: Antifouling technology- Past, present and future steps towards efficient and environmentally friendly antifouling coatings. Progress in organic coatings **50**. p 75-104.

Yebra D M, Kiil S, Weinell C E, Dam-Johansen K. 2006. Mini-review: Presence and effects of marine microbial biofilms on biocide –based antifouling coatings. Biofouling **22**. p 33-41.

Zhang X, Yang Z, Cai Z. 2008. The hydrogen peroxide impact on larval settlement and metamorphosis of abalone haliotis diersicolor supertexta. Chinese journal of oceanology and limnology **26**. p 238-241.

7. Chapter seven - conclusions and further work

Conclusions

In the preceding chapters the investigations into the potential of using enzymes to control the release of hydrogen peroxide and thereby facilitate the antifouling mechanism is presented. It is evident from the literature that several organisms are equipped with enzymes to protect them against hydrogen peroxide. Hydrogen peroxide is also occurring naturally in the marine environment, and to some extent produced within the organisms. However, it is also clear, that high quantities of hydrogen peroxide effectively kill many marine organisms. The feasibility of hydrogen peroxide-based antifouling is therefore very much dependent on how much hydrogen peroxide can be delivered. Furthermore, hydrogen peroxide can degenerate into hydroxyl radicals, which are very powerful oxidants. Therefore, the overall antifouling effect depends on the ratio of hydroxyl radicals to hydrogen peroxide, and the rate of degeneration into oxygen, which supposedly occurs via radical intermediates.

The applicability of peroxides of magnesium, calcium, strontium, and zinc peroxides as precursors for hydrogen peroxides in antifouling coatings has been tested during this work. The inorganic peroxides are compatible with coatings when in dry conditions, and the peroxides all leach from the coating in a controlled manner when mounted on a rotor in the laboratory. Polishing of inorganic peroxide-based coatings has also been documented. However, under seawater conditions, calcium-, magnesium-, and strontium peroxide fail mechanically. Therefore only zinc peroxide is suitable as ingredient in antifouling coatings and precursor for hydrogen peroxide. Under long term seawater exposure, the antifouling effect of a zinc peroxide-based fast polishing coating outperforms a zinc oxide-based reference in direct comparison. The improved antifouling ability of the zinc peroxide-based coating has been shown to be caused by the peroxide content of the coating. The average hydrogen peroxide release rate of 7.5 µg/(cm^2·day) over the eight months period, the experiment lasted is therefore responsible for the improved antifouling performance. However, from the results provided it is evident that zinc peroxide cannot compete with the more potent cuprous oxide-based antifouling that is commonly used today. It is therefore not likely that a zinc peroxide based coating will exceed a service life of 1 year. Furthermore, inorganic peroxides react with some organic molecules to give explosive components, and water-based technology is not compliable as water will induce the release of hydrogen peroxide. Therefore inorganic peroxide based antifouling is also concerned with safety issues.

Starch and hydrolytic enzymes have been used to provide seawater soluble alternatives to traditional polishing pigments (e.g. Cu_2O and ZnO) in antifouling coatings. The enzyme facilitates a slow conversion of water-insoluble starch into water-soluble glucose, and dissolution of glucose causes the development of a leached (porous) layer in the wetted, outermost part of the coating. Starch suitable for use as antifouling coating ingredient must contain little water-soluble material, only draw limited amounts of water into the coating, have small grain size and not agglomerate. The best starch-type amongst 13 different starch-types obtained from corn, rice and tapioca has been found to be the corn starch, C*gel 03401 from Cargill.

Spray drying together with glucoamylase starch granules with enzyme attached to the surface will be the result, and when this starch-enzyme pigment is enclosed in an antifouling coating matrix, leaching occurs in all the coatings tested, and polishing is detected for two of four binder systems investigated. Polishing rates of 7-10 µm/month, based on the enzymatic starch-degradation, have been measured, and references containing only starch (no enzyme) do not polish. Also, binder compositions that do not polish based on a high pigment content of cuprous oxide, polishes when the cuprous oxide is substituted for starch and glucoamylase. The polishing rates achieved are suitable for commercial ships, but they should be increased to meet yacht purposes. Enzyme activity in the coating may be changed to change the polishing rate of the coating, and the coating composition can also be optimised to minimise the adverse effects of the starch, and meet polishing requirements for yacht purposes.

An antifouling coating based on enzymatic conversion of starch into hydrogen peroxide has been tested for common coating properties and antifouling efficiency. Coatings have been formulated based on a non polishing binder system due to stability concerns. The coatings are shown to retain the requirements towards homogeneity and mechanical stability expected of an antifouling coating. However, the enzyme-mediated hydrogen peroxide release achieved is insufficient in terms of antifouling efficiency when tested under equatorial and Mediterranean climatic conditions. Under temperate (17 ºC) climatic conditions with corresponding low biofouling intensity, the coatings have earlier been described to provide antifouling protection. The difference in antifouling effect between the different types of climates has been very well explained by the temperature differences. It turns out that the stability of the release rates of hydrogen peroxide from the coatings is highly dependent on temperature, the higher the temperature, the more rapidly the hydrogen peroxide release rate drops. Also, the loss of hydrogen peroxide release rate is traced back to a loss in glucose release by glucoamylase. However, neither starch depletion, nor glucoamylase

stability of the native enzyme in hydrogen peroxide free environment can explain the rapid decrease in activity. Leaching assays based on rotary testing show that glucoamylase activity seizes more rapidly when hexose oxidase is present. Hydrogen peroxide is therefore concluded to be the cause of deactivation of the glucoamylase and a stable system delivering hydrogen peroxide for longer times can therefore only be obtained if glucoamyase is protected from hydrogen peroxide.

When releasing hydrogen peroxide from an inert surface directly into the sea, high release rates are required to keep the surface of a kraft paper totally free of fouling. It has been shown that during nine weeks immersion in seawater in Jyllinge, Denmark, fouling accumulates on a surface of kraft paper though hydrogen peroxide are released from the paper with a rate of approximately 224 µg/(cm^2·day). When 10 times that amount of hydrogen peroxide is released from a similar surface, the surface is completely fouling free during the full immersion period (nine weeks). Considerably more fouling is found on a reference not releasing any hydrogen peroxide at all. Hydrogen peroxide alone is not an antifouling agent remotely comparable to cuprous oxide. However it is proved that if released in high enough rates, hydrogen peroxide is very efficiently keeping surfaces clean of biofouling. The parameter limiting the applicability of hydrogen peroxide as antifouling agent is therefore whether the compound can be produced within an antifouling coating in these quantities. It is shown that neither inorganic, nor enzymatic generation of hydrogen peroxide is capable of delivering hydrogen peroxide in these quantities for more than a few weeks (depending on pigment volume concentration and dry film thickness). Hydrogen peroxide does therefore prevent biofouling, but very high release rates are required to keep surfaces fouling free. Hydrogen peroxide will therefore not be able to carry the required antifouling activity of a modern antifouling coating alone. However, it has also been shown that hydrogen peroxide significantly improves the antifouling activity of a polishing coating based on zinc oxide. Therefore, considerably lower release rates of hydrogen peroxide (7.5 µg/(cm^2·day)) is expected to contribute to biofouling protection (as opposed to deliver it all alone). Broad spectrum antifouling agents are often connected to adverse effects on the marine environment, due to toxicity. Therefore, environmentally benign antifouling will be depending on applying several environmentally friendly antifouling agents, and hydrogen peroxide from starch may become one, if the glucoamylase enzymes are optimised to withstand hydrogen peroxide. An advantage is that enzym-mediated hydrogen peroxide antifouling can be combined with metal free polishing, if glucoamylase is made sufficiently stable in the presence of hydrogen peroxide.

Further work

In order to be able to control polishing using hydrolytic enzymes and hydrolysable bioorganic material, the process of spray drying of the two components should be optimised. Mixing starch and glucoamylase in water will initiate the degradation of starch, resulting in water-soluble glucose that will eventually draw water into the coating and compromise its mechanical stability. Therefore parameters such as pH, and temperature (salinity will add to the water soluble content of the spray dried powder and should therefore be kept low) should be optimised so that the enzyme is as inactive in the slurry as possible. Furthermore, a spray drying process allowing for adding one of the two component as close to the nozzle as possible will further lower the amount of water soluble contaminants in the powder, and should therefore be developed.

The applicability of starch and glucoamylase in other binder systems and as a filler in antifouling coating for commercial ships should be tested to see if it is possible to lower the amount of cuprous oxide without compromising polishing rate (and antifouling activity). Furthermore, alternative enzymes and substrates should be tested, and finally the impact of substrate and products on the biofouling community should be investigated.

This work establishes that hydrogen peroxide in it-self is no measure against cuprous oxide as an active antifoulant, and if a single substitute for cuprous oxide is the aim, there should be no need to further look into hydrogen peroxide. However, hydrogen peroxide is shown to contribute to the antifouling activity of a coating that is already somewhat active (due to zinc oxide), and therefore hydrogen peroxide may be an environmentally friendly active ingredient in an antifouling coating based on several (environmentally friendly) antifoulants that together adds up to the level of cuprous oxide. The benefit of using enzyme-mediated hydrogen peroxide release will then be the possibility of inducing polishing independent on zinc oxide and cuprous oxide, and thereby control the release of compounds other than hydrogen peroxide. Should the coating-concept be further developed, however, then it is of key importance that the stability of glucoamylase in the presence of hydrogen peroxide is investigated and improved. However, it is not easy to simulate the conditions of the glucoamylase because the diffusion in the leached coating matrix is very different from those in a bulk solution.

Appendix

Appendix I - Hydrogen peroxide as antifouling agent: Fate in seawater and effect towards fouling organisms

Hydrogen peroxide (H_2O_2) is a potentially very attractive substitute for cuprous oxide. Its residence time in seawater is short, and it results in water and oxygen when degraded. Hydrogen peroxide is a strong oxidiser that is commercially available in various solutions. It is unstable, and decomposition results in water and oxygen (equation (I. I):

$$2H_2O_2(aq) \rightarrow H_2O(l) + O_2(aq) \quad \text{(I. I)}$$

Decomposition of hydrogen peroxide can be catalysed homogeneously or heterogeneously, and evidence points towards a free radical intermediate, implying that a chain reaction is involved (Eul et al. 2001). Compounds, catalysing homogeneous decomposition of hydrogen peroxide, include ferrous, ferric, cuprous, cupric, chromate, dichromate, molybdate, tungstate, and vanadate (Eul et al. 2001). Furthermore, hydrogen peroxide is decomposed by the enzyme catalase, and heterogen catalysis of the decomposition reaction occur with copper, mild steel, iron, silver, palladium, platinum, and oxides of iron, lead, nickel, manganese, copper, mercury and activated carbon (Eul et al. 2001). Hydrogen peroxide is also decomposed by alkaline impurities if pH is raised above 7; light and heat also affects the rate of hydrogen peroxide decomposition (Stringer 1993).

Hydrogen peroxide can react to give powerful radicals. In solutions and in catalysed reactions, the reaction giving two hydroxyl radicals pr hydrogen peroxide molecule predominates (c.f. equation (I. II)).

$$H_2O_2\,(aq) \rightarrow 2HO^{\cdot}\,(aq) \quad \text{(I. II)}$$

Hydrogen peroxide alone is a strong oxidant, with E0 of +1.8 V at pH=0 and 0.87 at pH=14. However, the hydroxyl radical is one of the most powerful oxidants known (Eul et al. 2001), and hydrogen peroxide together with a suitable catalyst is commonly applied as oxidation solution. Most well known is the Fenton solution, which contains iron together with hydrogen peroxide.

Hydrogen peroxide is used widely to control pollution because it results in water and oxygen and no toxic by-products (Eul et al. 2001), and at elevated concentrations (1-3%) hydrogen peroxide solutions are used as disinfectants (Eul et al. 2001). Hydrogen peroxide is “generally recognized as safe” (GRAS) in the US, the highest allowable level of treatment for food application is 1.25 wt% (Eul et al. 2001).

Hydrogen peroxide is widely present in nature, and can be regarded as nature's own disinfectant (Block 2001). It is known to function as a preservative in milk and honey, and it also exists in humans where it protects against infections. In natural waters, hydrogen peroxide concentrations vary a lot, some of the reported concentrations are summarised in (Stringer 1993). In surface seawater, the reported concentration ranges from 0.14 µg/l (North Atlantic) to 58 µg/l (Cheasapeake Bay). In Fresh waters concentrations of 0.34 µg/l (Lake Ontario/Erie/Jacks) have been reported as the minimum, and the maximum reported concentration (River Volga) is 109 µg/l (Stringer 1993).

The sources of hydrogen peroxide in natural waters are: UV induced reaction between organic suspended material and molecular oxygen, oxidation of iron or copper ions by molecular oxygen, deposition from rainwater, precipitation from atmosphere, and emissions caused by its use in domestic and industrial processes (Stringer 1993). The major source of hydrogen peroxide is abiotic photochemical processes (Petasne and Zika 1987).

Hydrogen peroxide sinks are redox reactions with suspended organic material or metals, decomposition by the catalytic action of enzymes (catalase, peroxidase) or self decomposition, which is catalysed by some metals (homogenic as well as heterogenic).

Hydrogen peroxide also occurs in living animals. When illuminated, the blue green algae produce between 1 and 50 µg/l·h (Zepp et al. 1987), and phytoplankton also forms hydrogen peroxide (Johnson et al. 1989). In the external environment of marine organisms, hydrogen peroxide is produced from metabolites excluded from the organisms (Stringer 1993).

In spite of a high release from industry and domestic sources, the presence of hydrogen peroxide in the environment is independent of these sources. This is due to the rapid decomposition of hydrogen peroxide in water. In fact, the amount of hydrogen peroxide in seawater deposited from rainwater is greater than the sum of industrial and domestic sources (Stringer 1993).

Hydrogen peroxide decomposes by a variety of processes in water, and decomposition of hydrogen peroxide in natural waters has been shown to generally follow first order reaction kinetics with respect to hydrogen peroxide concentration (Petasne and Zika 1987). Half-lives of hours have been predicted for freshwater. However, fresh water measurements do not apply to the oceans where the algae species differ, and there is considerably less suspended organic material (Petasne and Zika 1987). In freshwater algae and suspended materials play a major role in rapidly decomposing hydrogen peroxide (Petasne and Zika 1987).

The half-life of hydrogen peroxide measured in different waters has been summarised in (Stringer 1993), in lake water, the half-life is 7.8 h and increasing when the water is filtered. For filters with a diameter of 0.45 µm the half-life exceeds 24 hours (Stringer 1993). Furthermore, in seawater, the half-life of hydrogen peroxide varies depending on position. Half-lives range from less than 10 hours at coastal positions to 120 hours at surface waters in the open sea (Petasne and Zika 1987). Filtering the water increases the half-life considerably, and whereas autoclaving of the samples prevents hydrogen peroxide loss effectively, decomposition is accelerated by spiking the samples with bacteria. However, rupturing the cells by sonication, effectively killing cells, but retaining enzymatic activity, does not affect hydrogen peroxide decay (Petasne and Zika 1987). Therefore, biotic mediated hydrogen peroxide decomposition of small microorganisms predominates (Petasne and Zika 1987). However, Wong and Zhang report a catalytic effect of I^- on hydrogen peroxide in seawater (Wong and Zhang 2008). The hydrogen peroxide first oxidise I^- to I_2, and then I^- is regained by reduction with hydrogen peroxide, and a negligible auto-decomposition of hydrogen peroxide in seawater absent of iodine is reported (Wong and Zhang 2008). The fate of hydrogen peroxide in soil depends on microbiological flora and the presence of minerals in the soil. However, in all cases, the half-life is reported in minutes (Stringer 1993).

Hydrogen peroxide is active against bacteria, yeasts, fungi, viruses, and spores (Block 2001). It has been tested widely for its bacteriostatic effects, and it has been shown to have a more pronounce effect towards bacterial spores than germinated bacteria (Stringer 1993). Hydrogen peroxide affects the cytoplasm of biological cells (Denyer 1995) by Inhibition of cytoplasmic enzymes or interaction with functional bio molecules which causes Inhibition of catabolic and anabolic processes in the cell (Denyer 1995). Furthermore, hydrogen peroxide is an intermediate in oxygen metabolism, and to control the levels of toxic compounds, cells are equipped with catalase and peroxidases, enzymes that rapidly decompose hydrogen peroxide and superoxide (Petasne and Zika 1987). Catalase is an enzyme that very rapidly degrades hydrogen peroxide to water and oxygen, and its presence in cells ensures that bioaccumulation of hydrogen peroxide does not occur (Stringer 1993). Marine plankton and bacteria have been shown to contain peroxidase and catalase (Petasne and Zika 1987), and hydrogen peroxide decomposition in coastal water has been shown to predominantly be caused by these enzymes (Petasne and Zika 1987). Cells are protecting themselves from external sources of hydrogen peroxides and the radicals derived from hydrogen peroxide by adjusting the levels of catalase and peroxidase in the cells (Petasne and Zika 1987).

Copper toxicity towards some algae might be due to coppers inhibition of catalase by replacing iron in the enzyme (Stauber and Florence 1987).

The effect of hydrogen peroxide on bacteria is either via the hydroxyl radical or enzyme catalysed reaction to hypochlorite (Block 2001). The bactericidal effect of hydrogen peroxide is thought to be caused by its oxidation of sulfhydryl groups and proteins (Block 2001). In fact, hydroxyl radials are suspected to be the reason for biological damage of irradiation (Block 2001). The reported effects of hydrogen peroxide on aquatic organisms is summarised in (Stringer 1993). The marine algae, Nitzchia closterium has been reported to be affected by hydrogen peroxide concentrations as low as 0.85 mg/l (Stringer 1993). However to see effects on the freshwater alga, *Anabaena*, hydrogen peroxide concentrations of 9.86 mg/l are required (Stringer 1993).

The reported effects of hydrogen peroxide as antifouling compound that is available in peer reviewed literature are summarised in Table I. I. According to (Elzanowska et al. 2000) hydrogen peroxide alone is not very toxic to prokaryotic cells. However, adding low-valent transition metal ions like ferrous, cuprous salts or ferric complexes or cupric ions improve the hydrogen peroxide oxidative degradation of various biological compounds. Hydroxyl radical is claimed to be the actual oxidant. In metal ion depleted media, relatively high concentration of hydrogen peroxide is toxic to biofouling. The toxic concentration is reduced sharply (about hundred fold) in the presence of metal salts. Therefore, high hydrogen peroxide concentrations are presumably required for killing in the absence of metal ions (Elzanowska et al. 2000).

Hydrogen peroxide together with ferric ions, inhibit mussels (*Mytilus galloprovincialis*), polychaetes (*Hydroides norvegicus, Dexiospera foraminosa*), brozoa (*Bugula dentate*), hydroid *(Obelia*) and colonial tunicates from settling (Nishimura et al. 1988). Barnacle larvae (*Balanus amphitrite*) are not affected during settling; however, after metamorphosis, the barnacle growth-rate is halved. The difference in the effect of hydrogen peroxide seen on barnacles before and after metamorphosis is presumably due to the difference in feeding. When in the larvae stage, barnacle does not feed, but nourish on the nutrients conserved in their body (Nishimura et al. 1988). The effectiveness of hydrogen peroxide towards settling of marine biofouling organisms therefore vary with the species of the biofouling organisms and depends on the biofouling organisms mobility, time limit of settlement, feeding status and tolerance to chemicals (Nishimura et al. 1988).

A concentration of 0.07mg/L hydrogen peroxide is reported to inhibit biofouling by mussels significantly, and addition of iron sulphate improves the effect. With a combination of hydrogen peroxide and iron sulphate, biofouling is inhibited to 1/6 of the control (Ikuta et al. 1988).

According to (Zhang et al. 2008), settlement and metamorphosis of barnacles is unaffected by hydrogen peroxide concentrations of up to 13.6 mg/l. The settlement increases for concentrations in the range between 13.6 and 27.2 mg/l, but decreases when the hydrogen peroxide concentration exceeds 34mg/l (Zhang et al. 2008).

Electrochemical generation of reactive oxygen species is reported very efficient in laboratory antifouling assays (Perez et al. 2008). Catalase in the solution inhibits the effect of hydrogen peroxide, which indicates an important effect of hydrogen peroxide. A concentration of 13.6 mg/l hydrogen peroxide inhibits biofouling effectively. However, pulsed electric frequency inhibits biofouling more potently at lower hydrogen peroxide concentrations, and therefore, hydrogen peroxide seems to be a precursor for the actual antifouling agent, which supposedly is the hydroxyl radical (Perez et al. 2008).

Precursor inorganic peroxides have been used in hydrogen peroxide based antifouling (Olsen et al. 2008). Magnesium-, calcium-, strontium-, and zinc –peroxides are used to generate hydrogen peroxide in-situ in an antifouling coating. However, only coatings based on zinc peroxide are mechanically stable for long term seawater experiment. In a direct comparison, zinc peroxide based antifouling outperforms a similar coating based only on zinc oxide. This effect is achieved by a hydrogen peroxide release rate from the zinc peroxide based coating of 7.48 µg/(cm^2·day) corresponding to(0.22 µmoles/(cm^2·day) (Olsen et al. 2008).

Photocatalytic hydrogen peroxide generation in-situ in antifouling coatings is described in (Morris and Walsh 1999). Zinc oxide coated with photoactive material (anastase) generates hydrogen peroxide from oxygen when illuminated with UV light. The authors report release rates corresponding to 1.26 µg/cm^2·d corresponding to 0.037 µmoles/(cm^2·d) of hydrogen peroxide in continuously aerated water. However, no estimation of antifouling effect is available in the patent.

Enzymatic generated hydrogen peroxide for antifouling coating purposes has also been patented (Poulsen and Kragh 2002). Molecular oxygen is used to oxidise glucose to gluconolactone under the release of hydrogen peroxide. The reaction is catalysed by hexose oxidase. Another enzyme (glucoseamylase) is used to provide glucose from starch in a hydrolytic reaction. The patent report measurable hydrogen peroxide release from formulated coatings, and claims the composition capable of preventing biofouling. However, data supporting the antifouling potential is not submitted (Poulsen and Kragh 2002).

Table I. I: Summary of the reported effects of hydrogen peroxide on antifouling animals. The references are ordered chronologically.

Effective amount of H_2O_2	Test	Species	Reported effect	Reference
57.8- 578 mg/l	Chlorophyll reduction by bulk solution concentration.	Freshwater algae	Chlorophyll reduction to below 5 wt% after 24 hours.	Kay et al. 1982
1.36 mg/l, 1.7 mg/l	Cells exposed to the biocides in bulk solution for 24 h. Cell division rates monitored.	*Nitzchia closterium*, *Chlorella pyrenoidosa*. Marine algae	Cell division rate reduced to below 10 %. No effect on photosynthesis was identified.	Stauber and Florence 1987
0.7-1 ppm (35% H_2O_2 solution)	Presence of organisms on a net in a collector (test tube) with a water flow through. 90 days growth time.	Water taken from the sea. Abryozoa, Tunicates mussel polychaetes and malacostracans most abundant	Up to 90% settlement inhibition of the predominant species. Polychaetes and barnacle unaffected. Addition of iron(II) improve performance.	Nishimura et al. 1988
0.5-1.4 ppm (35% H_2O_2 solution)	Presence of organisms on a net in a collector (test tube) with a water flow through. 90 days growth time.	Water taken from the sea.	Reduction of organisms to 17.8 – 6.8 % of the control. Growth rate of mussels significantly inhibited at 2 ppm (0.35wt% solution). Fe(II) species improve the effect.	Ikuta et al. 1988
34 mg/l	Metamorphosis and settlement under laboratory conditions. Bulk solution H_2O_2 concentrations.	*Haliotis diversicolor*, abalone	Metamorphosis completely inhibited at 1 mM. Settlement approximately halved. Between 0.4 and 0.8 mM settlement and metamorphosis improved with increasing H_2O_2 concentrations.	Zhang et al. 2008
13.6 mg/l	Barnacle larvae settlement in bulk solution concentration assay. Experiment ended when 40% had settled.	Larvae from *Balanus amphitrite* (Barnacle)	0.4 mM hydrogen peroxide inhibits settlement. Catalase inverse the effect, pulsed electronic frequencies has better effect at lower hydrogen peroxide concentrations.	Perez et al. 2008

It is evident that hydrogen peroxide does affect a wide range of biofouling species. However, it is also obvious that some are more robust towards hydrogen peroxide than others. Given that biology has provided means for organisms to protect themselves against hydrogen peroxide, it seems obvious that concentrations exceeding naturally occurring hydrogen peroxide levels considerably, must be provided to achieve a broad spectrum, long term antifouling effect of a coating.

Interestingly, there seems to be consistency that the effect of hydrogen peroxide as an antifouling agent can be improved if catalysts, promoting its decomposition into reactive radicals are applied as well (Perez et al. 2008), (Ikuta et al. 1988), and (Nishimura et al. 1988). As described above, biotic hydrogen peroxide decay is the predominant way of decomposition of hydrogen peroxide. This is done by the catalase-positive organisms, and as catalase is the only decomposition path of hydrogen peroxide that almost certainly do not deliver radicals to the environment (Boon et

al. 1997). Therefore, converting the hydrogen peroxide to the more powerful hydroxyl radical before it interacts with the biofouling organism should improve the overall antifouling effect. However, the reactivity of the hydroxyl radicals are greater, and therefore, they are also expected to be less stable. Therefore, when not present as bulk solution species, the overall loss of reactive oxygen species before it meets the target organism might be increased by adding catalyst to a coating. Complete loss of the effect of active hydroxyl radicals after 2 minutes from the spot of its introduction has been reported (Ikuta et al. 1988). This indicates low stability of the hydroxyl radicals, and if hydrogen peroxide should be produced in-situ in an antifouling coating, there is little doubt that it will be in a leached layer of the coating, and require transport by diffusion before the active agent reaches its target organism. The importance of the hydroxyl radical/hydrogen peroxide reaching its target organism before it is decayed into oxygen goes beyond the loss of antifouling effect; the oxygen may promote biofouling by aerobe organisms, and molecular, gaseous oxygen may stress the coating leading to coating failure. It is therefore not obvious that when provided in-situ in an antifouling coating, hydroxyl radicals is better than its parent compound, hydrogen peroxide. Another consideration to take into account is the possibility of the radical to react with the binder system on its way through the leached layer.

All the reported effects of hydrogen peroxide and its reactive derivatives have been based on bulk solution concentration studies. It is important to distinguish between the results obtained from assays with the organisms present in a solution of the active compound, and the potential effect of a flux or release rate of the compound. What biofouling animals by the side of a ship hull will experience is a continuous release of the active ingredient and not an ocean with a given concentration, and lifetimes of active compounds should be compared with diffusion lengths, when the compounds are as short lived as hydroxyl radicals. Furthermore, to be able to formulate coatings, it is necessary to know the amount of ingredients needed during paint production. Will the required amount of active ingredient be technically feasible? Will critical PVC be exceeded? And will the needed amounts be compatible with the binder? For potential water soluble active coating ingredients, these questions are best answered from efficient release rate measurements.

Hydrogen peroxide can be produced in-situ by a number of techniques. Enzymatic oxidation of glucose, hydrolysis of inorganic peroxides, and reaction between UV-light and special pigments are some. Enzymatic generation of hydrogen peroxide is believed to be a promising method of environmentally friendly antifouling. Inorganic or organic peroxides can be used, and in situ

generation based on catalytical inorganic pigments and UV-light is used. Table I. II show the pros and cons of the different approaches of hydrogen peroxide generation in antifouling coatings.

Table I. II: Pros and cons of the three reported means of providing hydrogen peroxide in-situ in antifouling coatings

Method	Pros	Cons
Enzymatic generation	No peroxide in coating system during production and storage Possible to control release rate by enzyme manipulations	Low peroxide number pr volume coating. Enzymatic decay. Combination of enzymes and organic solvent or H2O2 production during storage.
Inorganic/organic peroxides as precursors	High peroxide number pr volume coating.	Hazardous properties with some organic compounds Oxidation of binder material Stability of the compounds during storage (High solubility)
In situ generation (UV-light)	No peroxide in coating system during production and storage High peroxide number pr volume coating. Possible to control release rate by catalytic pigment content.	Limited access of UV-light on ship hulls.

UV interaction is already applied, but the major disadvantage, limited UV-light beneath sea water surface, seems as a large obstacle to be overcome for this technology to reach the broader market. Therefore this work will be dedicated towards providing hydrogen peroxide from antifouling coatings by means of enzymatic hydrolysis of starch and subsequent oxidation of glucose, and by use of precursor inorganic peroxides.

References

Eul W., Moeller A., Steiner N. 2001. Hydrogen Peroxide. Kirk-Othmer Encyclopedia of Chemical Technology. p 1-53.

Stringer, D A, (Editor). 1993. Joint assessment of Commodity Chemicals No. 22 – Hydrogen peroxide CAS No. 7722-84-1. European Centre for Ecotoxicoloy and Toxicology of Chemicals.

Block, S S (editor). 2001. Disinfection, Sterilization and Preservation. Fifth edition. Lippincott Williams & Wilkins.

Petasne R G, Zika R G. 1987. Hydrogen peroxide lifetimes in south florida coatsal and offshore waters. Marine Chemistry **56**. p 215-225.

Zepp R G, Skurlatov Y I, Pierce J T. 1987. Algal-induced decay and formation of hydrogen peroxide in water: Its possible role in oxidation of anilines by algae. In: Cooper Jr., WJ (editor). Photochemistry of environmental aquatic systems. American chemical society. p 215-225.

Johnson K S, Willason S W, Wiesenburg D A, Lohrenz S E, Arnone R A. 1989. Hydrogen peroxide in the western Mediterranean Sea: a tracer for vertical advection. Deep sea research par A oceanograpphic research papers **36**(2). p 241-254.

Wong, G T F, Zhang L-S. 2008. The kinetics of the reactions between iodide and hydrogen peroxide in seawater. Marine Chemistry **111**. Pp 22-29. (2008)

Denyer S P. 1995. Mechanism of action of antibacterial biocides. International biodeterioration & biodegradation. p 227-245.

Stauber J L, Florence T M. 1987. Mechanism of toxicity of ionic copper and copper complexes to algae. Marine biology 94 (511). p 511-519.

Elzanowska H, Wolcott R G, Hannum D M, Hurst J K. 2000. Bactericidal properties of hydrogen peroxide and copper or iron-containing complex ions in relation to leukocyte function. Free radical biology & medicine **18**. p 1-86.

Nishimura K, Yasunaga T, Ichikawa S, Wakao Y. 1988. Development of a new antifouling method for a marine cooling water system. Marine biology. **99**. p 145-150.

Ikuta S, Ichikawa S, Wakao Y, Nishimura K, Yasunaga T. 1988 " Inorganic peroxides for heat exchanger biofouling control." The american society of mechanical engineer .

Zhang X, Yang Z, Cai Z. "The hydrogen peroxide impact on larval settlement and metamorphosis of abalone haliotis diersicolor supertexta." Chinese journal of oceanology and limnology **26**. Pp 238-241. (2008)

Perez R, Noguera D, Anderson M. 2008. Localized generation of reactive oxygen species by pulsed electric signals and their effect on biological adhesion. Poster on the 14th international congress on marine corrosion and fouling.

Olsen S M, Pedersen L T, Hermann M H, Kiil S, Dam-Johansen K. "Inorganic precursor peroxides for antifouling coatins." Journal of coatings technology and research. (2008). (Paper has been accepted, but publication is pending).

Morris S R, Walsh M A. 1999. Zinc oxide photoactive antifoulant material. USpatent 5,916,947.

Poulsen C H, Kragh K M. 2002. Compostion. US patent application US2002/0106361A1.

Boon E M, Downs A, Marcey D. 1997. Catalase: H_2O_2: H_2O_2 oxidoreductase. Biomolecules at Kenyon. Accesed October 2008 at http://biology.kenyon.edu/B

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